THIS NUMBER COMPLETES VOLUME 11

EXPERIMENTAL CELL RESEARCH

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VOLUME 11 · DECEMBER 1956 · NUMBER 3

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N. Y.

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Experimental Cell Research **11**, *511–519 (1956)*

ALKALINE PHOSPHATASE AND CALCIFICATION IN TUBERCULOUS LYMPH NODES

E. HENRICHSEN

Biological Institute, Carlsberg Foundation, Copenhagen, Denmark

Received May 5, 1956

In a previous paper it was reported that alkaline phosphatase in the cytoplasm of fibroblasts, cultivated *in vitro*, occurs simultaneously with the degeneration of the cells [5]. Further, a relationship was described between the appearance of alkaline phosphatase, cellular alterations (degeneration, cell death) and of calcification [6]. It was demonstrated that (1) calcification in tissue cultures of fibroblasts appeared in areas with alkaline phosphatase activity, (2) calcification did not take place when the phosphatase-containing cells were still living, whereas it occurred when the phosphatase-containing cells died, as indicated from lacking nuclear staining by methyl green, and (3) further calcification would take place afterwards, at a time when alkaline phosphatase was no longer present.

A hypothesis was submitted on the role of alkaline phosphatase in calcification based on the assumption that alkaline phosphatase reacts in different ways depending on whether it is present in a living or in a dead cell.

Histochemical investigations on the localization of alkaline phosphatase in tuberculous lesions were previously performed by Takeuchi and Takamatsu [15] and by Gomori [4]. Alkaline phosphatase was demonstrated in degenerating cells, but not after complete necrosis. Gomori connected alkaline phosphatase with calcification, but did not discuss his findings in relation to the two factors in Robinson's theory of calcification.

The purpose of the present investigation is to examine whether the above-mentioned relationship in tissue cultures between alkaline phosphatase, cellular alterations and calcification also occurs in tuberculous lymph nodes. This tissue represents a pathological calcification *in vivo*. The relation of calcification to other staining phenomena which have been connected with calcification will also be discussed.

MATERIALS AND METHODS

Mesenterial lymph nodes from pigs with signs of tuberculous infection (avian type) were collected by veterinarian students when following a course of meat control at

the slaughter house at Ringsted and the destruction factory in Ortved. The lymph nodes were cut in slices and placed in 96 per cent alcohol for 24 to 72 hours, depending on the time spent on their way to the laboratory. Because of the long alcohol treatment alkaline phosphatase was inactivated in some of the lymph nodes. These samples were discarded as indicated below. The material used consisted of 28 lymph nodes.

The specimens were embedded in paraffine at 56°C via alcohol and xylene and cut in serial sections. The slides were stained for alkaline phosphatase by the Manheimer–Seligman method [11] and for calcium phosphate deposits by the von Kossa method as described by Lillie [10]. Haematoxylin–eosine stainings, van Gieson–Hansen's collagen staining and methylgreen–pyronin staining (Leuchtenberger's technique [8]) were also used. Stainings with toluidine blue, McManus–Hotchkiss periodic acid–leucofuchsin staining (PAS staining) and the Feulgen staining were used for special purposes. Glycogen was demonstrated by the PAS method as indicated by Pearse [12]. Control sections were stained in the same way after treatment with malt diastase.

RESULTS

Normal lymph nodes.—In lymph nodes with no signs of tuberculous infection alkaline phosphatase was present in the small vessels and in some of the cells. After the red dye of the Manheimer–Seligman staining had been removed with alcohol and after staining with Giemsa stain, the eosinophile leucocytes were found to show positive phosphatase reaction. Some cells in the walls of the vessels and in the fibrous stroma also gave a positive reaction for phosphatase. A comparison with toluidine blue stained sections showed that it was the mast cells which gave the reaction for alkaline phosphatase (Fig. 1). This finding is in accordance with the results of Dalgaard and Dalgaard [2]. Besides the mast cells no other cells in the fibrous stroma showed alkaline phosphatase activity, a finding which seems to be contrary to the findings of Manheimer and Seligman.

When the normal staining for alkaline phosphatase activity, as described here, was not found in a lymph node, it was assumed that alkaline phosphatase had been inactivated during a prolonged fixation in alcohol, and the sample was discarded.

McManus' stainings revealed some faintly stained PAS positive material in the fibrous stroma, but almost nothing in the stroma of the lymph follicles. Glycogen was never demonstrated in normal lymph nodes. Toluidine blue staining showed a strong γ-metachromasia of the mast cells. A weak β metachromasy was found in the ground substance of the connective tissue, but also in many nuclei, especially of the lymphocytes. Only the nuclei were stained by the Feulgen method and by methyl green. Positive pyronin

staining was found in the cytoplasm of the connective tissue cells and to a lesser degree in the cytoplasm of the lymphocytes.

Tuberculous lymph nodes.—In the earliest stages of the tuberculous lesion, alkaline phosphatase is present in the center. When necrosis takes place in the center, there is no more alkaline phosphatase. As the process spreads and the lesion grows in size, alkaline phosphatase is found as a red stained ring in the periphery of the tuberculous lesion (Fig. 2). The phosphatase is always localized to cells in an early state of degeneration, but when the degeneration is complete and the cells become necrotic, phosphatase is no longer present in this part of the lesion. Therefore the phosphatase apparently moves peripherally as the lesion grows in size. When sections stained for phosphatase are compared with those stained with haematoxylin–eosine, methylgreen–pyronin and with slides stained by the Feulgen method, the nuclei of the phosphatase-containing cells are found to be pycnotic with condensed nuclear staining. This differs from the findings in tissue cultures of fibroblasts, where the phosphatase-containing and degenerated cells showed signs of hydropic degeneration with a gradual fading of the nuclear staining reactions. In the most central part of the phosphatase-stained area the cells show signs of heavy degeneration or cell death. The nuclei are broken into smaller pieces and methyl green and Feulgen stainings demonstrate that DNA is present in the smaller sections. In the actual necrotic area, more in the center, no nuclear material is to be detected (Fig. 3).

Calcification has never been demonstrated in the most peripheral areas of the tuberculous lesions, where the phosphatase-stained cells were not necrotic. More centrally calcification appeared localized to single necrotic cells, some of which were still stained for alkaline phosphatase. In the central, actual necrotic area calcification was as a rule more solid and was no longer localized to the single cells (Fig. 4). As mentioned above, no phosphatase could be demonstrated in this part of the tuberculous lesion.

Outside the phosphatase stained area, circular running fibrils are seen in the connective tissue. Phosphatase is not present here, but PAS staining reveals many granules of glycogen in the connective tissue cells (Fig. 5). Glycogen is still found in some cells in the periphery of the area which contains the phosphatase-stained cells; in the centre there is no glycogen, but PAS positive stained material is found in increasing intensity towards the calcified necrotic area. Treatment with hyaluronidase does not alter this staining reaction.

Toluidine blue stainings showed a weak β-metachromasia in the necrotic area. This was seen in the normal material and in many nuclei outside the

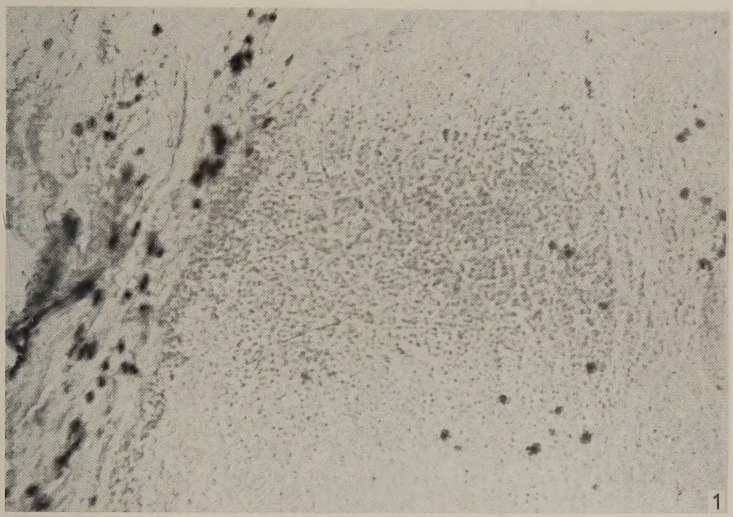
tuberculous lesion. This metachromasia disappeared immediately by mounting in glycerol jelly or by treatment with alcohol and mounting in Depex. Previous treatment with hyaluronidase did not influence this metachromatic staining.

DISCUSSION

The appearance of alkaline phosphatase in the lymphocytes upon degeneration due to tuberculous lesion indicates that the cells of the lymph node behave in the same way as fibroblasts from chick embryo hearts cultivated *in vitro*. Both cell types pass through a state in which alkaline phosphatase appears in the cytoplasm in the course of the alterations from normal to dead cell, when the process takes some time. From the experiments of Gomori [4] it is known that alkaline phosphatase does not appear when necrosis is made by injecting alcohol into animal tissues. The present author has performed similar experiments during preliminary work with bone induction by use of alcohol extracts of bone marrow, as described by Levander [9]. In these experiments no alkaline phosphatase appeared in the necrotic areas. Gomori assumed that the phosphatase was absorbed by the cells during development of necrosis and again liberated when the cells were totally necrotic. From experiments in tissue cultures we know that this cannot be the explanation, since alkaline phosphatase activity appears also in cultures in which the phosphatase of the medium is destroyed by heating [5].

The relations between alkaline phosphatase, calcification and cell death which have been found in the present material are in accordance with the findings in tissue cultures. The type of degeneration in the two materials differs. In fibroblasts cultivated *in vitro* the degenerated cells showed hydropic nuclei with a gradually disappearing staining ability. In the tuberculous lymph nodes pycnosis with increased staining was found. At the time when the initial calcification of the single cells sets in, alkaline phosphatase disappears and further calcification takes place at a time where no phosphatase is present in the necrotic area.

Robison and Rosenheim [13] suggested that two factors were active in calcification, namely the alkaline phosphatase and a local (or second) factor. The local factor was assumed to cause precipitation of calcium phosphate when the solubility product of this salt was exceeded by accumulation of phosphate ions as a consequence of the action of alkaline phosphatase on phosphoric esters. In this way the absence of calcification—for example in kidney tissue, which contains alkaline phosphatase in the tubulus cells—was explained as being caused by lack of the local factor.



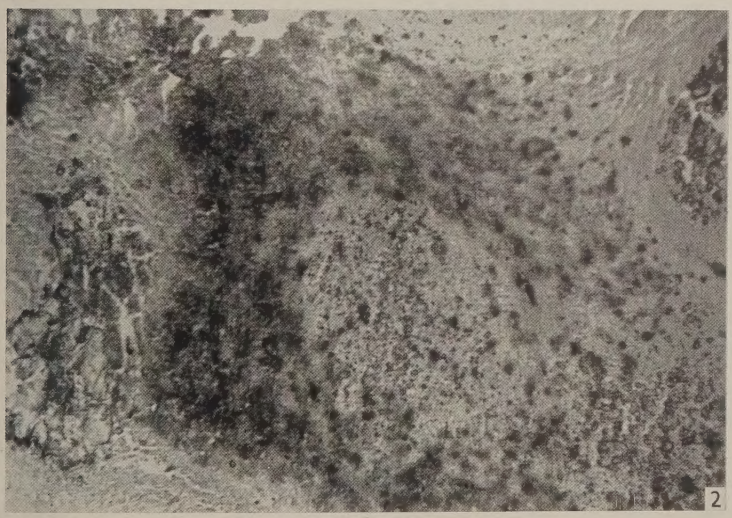
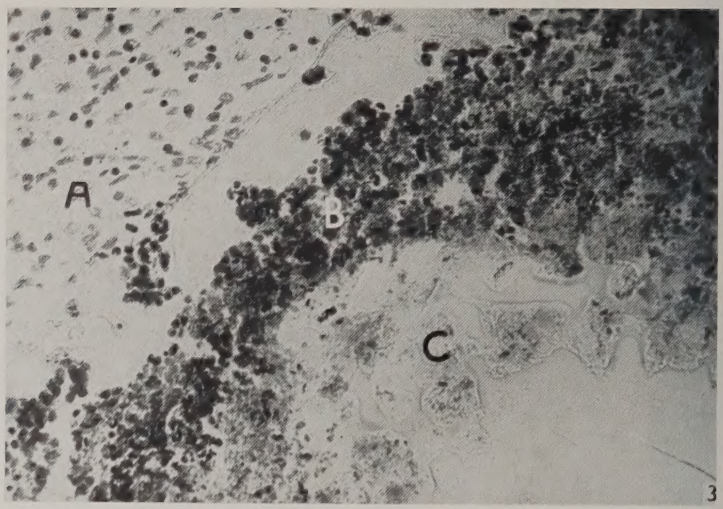


Fig. 1. Section of normal lymph node. Manheimer–Seligman staining for alkaline phosphatase. Eosinophile leucocytes (right) and mast cells (left) are stained for alkaline phosphatase. × 285.
Fig. 2. Section of tuberculous lymph node. Manheimer–Seligman staining for alkaline phosphatase. Note the ringshaped localization of the phosphatase stained cells. × 285.



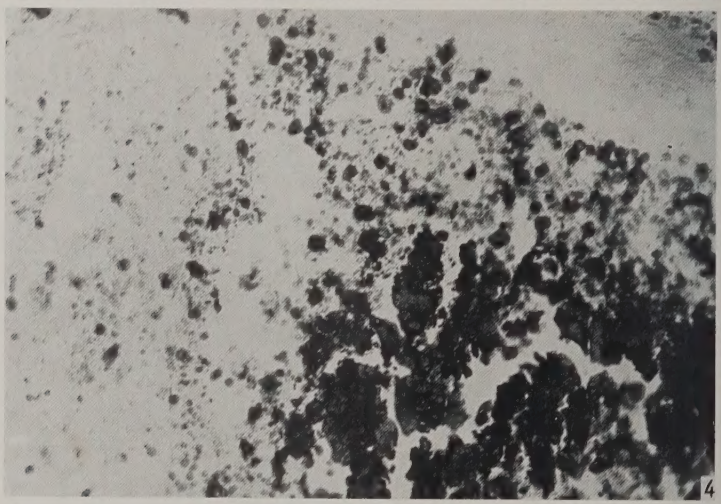


Fig. 3. Section of tuberculous lymph node. Feulgen staining. Part of tuberculous lesion with surrounding connective tissue (*A*). The cells in the connective tissue show normal nuclear staining. In the periphery of the lesion (*B*) the nuclei are pycnotic with increased staining. In the necrotic area (*C*) no nuclear staining are seen. × 475.
Fig. 4. Section of the same lymph node as Fig. 3. Von Kossa staining. In the periphery of the tuberculous lesion the calcification is localized to single cells. In the central part of the lesion the calcification is more solid and calcification is found outside the cell boundaries. × 475.

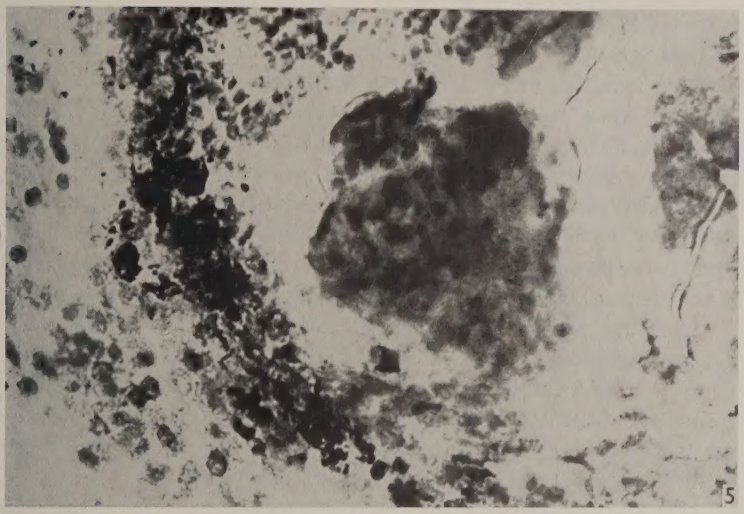


Fig. 5. Section of tuberculous lymph node. PAS staining for glycogen. Part of tuberculous lesion with surrounding connective tissue. Note that the glycogen-loaded cells are localized in the peripheral part of the tuberculous lesion and in the innermost part of the connective tissue. × 475.

Following this hypothesis the phosphatase-containing degenerated cells in the periphery of the tuberculous lesion, where no calcification is found, only contain the first factor—the alkaline phosphatase—but not the second or local factor. The second factor should then be present more centrally in the tuberculous lesion, where calcification takes place. The most striking morphological difference between these two localizations is that the phosphatase-containing cells are dead in the area where calcification occurs. This was also the case in tissue cultures of fibroblasts. This observation was used as an explanation in a revision of Robinson's phosphatase theory of calcification. The same relationship between alkaline phosphatase, cell death and calcification as seen in tissue cultures of heart fibroblasts is also found in this material, and the observation therefore strengthens the hypothesis.

The presence of a PAS positive, metachromatically stainable material in calcifiable tissues has been connected with a local factor in the process of calcification [7, 14]. It was suggested that chondroitin sulfuric acid or another mucopolysaccharide was responsible for this staining. On the basis of a parallel drawn from the chondroitin sulfuric acid in cartilage this material

was suggested as necessary for the formation of calcium phosphate crystals. The findings presented here lead to the suggestion that the β-metachromasia found in the necrotic area is the same material which stains metachromatically in the normal nuclei and may therefore be nucleic acids. Fischer [3] has shown that DNA and RNA are able to stain metachromatically with toluidine blue and so have Bank and Bungenberg de Jong [1], Weismann *et al.* [16] and Wislocki *et al.* [17]. The PAS positive material could be split products of glycogen, present outside the area of phosphatase-stained degenerated cells, thus resembling the glycogen in cartilage. Alkaline phosphatase is able to dephosphorylate some of the split products of glycogen which are formed by phosphorylative glycogenolysis and to deliver the phosphate ions required for local calcification in a high concentration. In necrotic areas depolymerized RNA might be another organic phosphate dephosphorylated by alkaline phosphatase. It is not possible to determine RNA in the necrotic area, as the pyroninophile material present does not change subsequent to RNAse treatment. Dephosphorylated RNA must be able to stain by the PAS method, as it now contains a diol [12]. It is possible that part of the PAS positive material in the necrotic area consists of dephosphorylated RNA. If this is the case, the PAS positive material, as suggested by Levine *et al.*, is related to the calcifying mechanism since it originates from the ribonucleic acids serving as substrate for alkaline phosphatase. Metachromasia was caused by nucleic acids, and there is no reason to believe that these two staining procedures show anything but results from the necrotic process, which leads to calcification owing to the action of the alkaline phosphatase liberated from the necrotic cells.

SUMMARY

1. The relationship between alkaline phosphatase, cell degeneration, cell death and calcification in tuberculous lymph node has been studied.

2. Alkaline phosphatase appeared in the cells during degeneration; calcification took place when the phosphatase-containing cells became necrotic and calcification proceeded at a time when the phosphatase had disappeared. This relationship is the same as that seen in cultures of fibroblasts.

3. Glycogen formation was the first noticeable cell reaction to the tuberculous lesion. Its disappearance at a time when phosphatase is present seems to parallel the findings in cartilage.

4. The relationship between a PAS positive, metachromatically stainable material and a local factor, necessary for the process of calcification, has been

doubted. It is shown that the metachromatically stainable material in the calcified areas in tuberculous lymph nodes can be nucleic acids and that the PAS positive material is possibly dephosphorylated depolymerized RNA.

My thanks are due to Dr. Albert Fischer for his interest in this work. The helpful advice of Dr. Tage Astrup in preparing the manuscript is gratefully acknowledged.

This investigation was supported by grants from Landsforeningen til Kræftens Bekæmpelse and from Statens Almindelige Videnskabsfond.

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CONCENTRATION OF RNA ON THE HETEROPYCNOTIC XY BIVALENT OF THE RAT

S. OHNO, W. D. KAPLAN, and R. KINOSITA

Department of Cytology and Genetics, Division of Research, City of Hope Medical Center, Duarte, Calif., U.S.A.

Received May 20, 1956

The peculiar heteropycnotic behavior of the sex chromosomes during meiosis in the male was observed and described long before the significance of these chromosomes in relation to sex determination was understood. The term X chromosome was originated by Henking, who as early as 1891 observed the greatly condensed and deeply staining "peculiar chromatin-like element" which was in marked contrast to the other thread-like and lightly staining chromosomes of the meiotic figures in the hemipteron *Pyrrhocoris*. Arriving at no very clear idea of the nature of this element, he labeled it "X". Subsequently heteropycnosis has been shown to be a widespread characteristic of the sex chromosomes. Most recently the heteropycnotic nature of the sex chromosomes has been re-described in man [4], in the mouse [5] and in the rat [6].

Recently we have found that the heteropycnotic XY bivalent of the rat is embedded in a vesicle which contains an appreciable amount of RNA. Since a concentration of RNA around the heteropycnotic XY chromosomes may indicate a metabolic significance for this peculiar behavior of the sex chromosomes, it was thought worthwhile to report the following findings.

EXPERIMENTAL

Rat testes were obtained from the Wayne Pink Eye, Long Evans and Japanese albino strains, inbred in our laboratory. Morphological studies of the first meiosis were made on fresh cells using phase microscopy or on smear preparations fixed and stained by our previously described technique [3]. Feulgen staining was sometimes used in addition to our 1 *N* HCl-picric acid Giemsa staining. The cytochemical studies were made on Carnoy fixed materials imbedded in paraffin. Sections were cut 6 micra thick; occasionally at 2 micra. The cytochemical determination of DNA was made by Feulgen staining and that of RNA was made either by staining with 1 per cent thionin solution or by the methyl green-pyronine staining of Unna–Pappenheim before and after digestion with ribonuclease. The substance which lost its

metachromasia or pyroninophilia after 90 minutes of incubation with the ribonuclease solution (10.3 mg recrystallized Armour ribonuclease in 100 cc pH 5 acetate buffer solution) at 37°C was determined as RNA.

OBSERVATIONS

The study is based upon the examination of a total of approximately 200 nuclei.

Preleptotene stages.—There appear to be no generally recognized terms for the earliest stages of the growth period of the rat primary spermatocyte. Thus, consistent with terminology set forth in Wilson [7] the preleptotene stages described here refer to those preceding the appearance of the typical leptotene nucleus.

Condensation of the sex chromosomes appears to begin soon after the final spermatogonial telophase. Thus, from the very beginning of the meiotic prophase, the primary spermatocyte possesses a strongly condensed Feulgen-positive body on the inner surface of its nuclear membrane. The nucleus during preleptotene stages has two or three medium-sized nucleoli, in contrast to the spermatogonial nucleus which, as a rule, has a single large nucleolus. The heterochromatic XY is found always on the nuclear membrane, while the nucleoli are present in the interior of the nuclear sphere. The heteropycnotic XY has never been found in association with a nucleolus.

In the nuclei of the smallest primary spermatocyte, thus at the pre-leptotene stage of development, the individual chromosomes, other than the heteropycnotic XY are not distinguishable but present the appearance of a net-like framework resembling somatic interphase nuclei. Typical features of this early stage are presented in the phase photomicrograph of the living primary spermatocyte (Fig. 1). The heteropycnotic XY may be seen as a dark rod-shaped body on the nuclear membrane. Apart from this, three grayish spherical bodies, apparently nucleoli, are present in the interior.

The term "prochromasome stage" appears best to represent the features of the nucleus at the next stage of the pre-leptotene period. Wilson's [7] description of this condition eminently fits the picture observed here. "The nuclear framework ... draws together into separate, more or less massive chromosome-like bodies." The contrast between the heteropycnotic XY bivalent and the autosomes is the least marked at this stage (Fig. 2). In this Feulgen-stained nucleus the heteropycnotic XY is barely recognizable as a short plump body on the nuclear membrane. The other chromosome-like bodies are as intensely Feulgen positive as the heteropycnotic XY. Again the

XY bivalent is in no way connected with either of the two Feulgen-negative nucleoli in the interior.

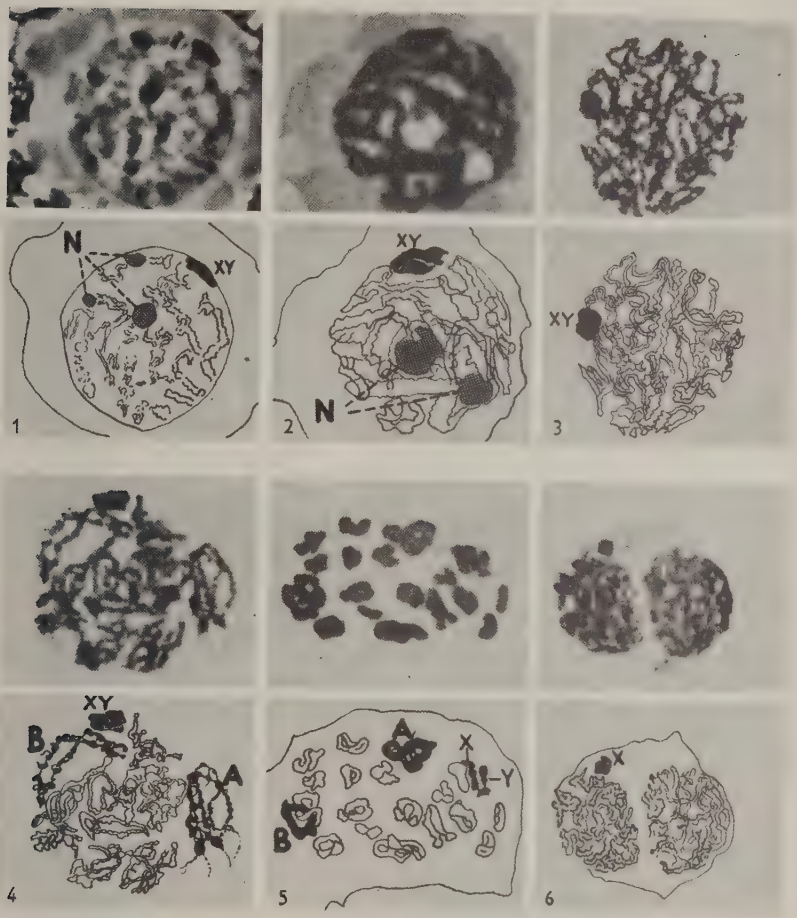
Cytochemical studies of pre-leptotene nuclei were carried out on paraffin-sectioned material. These have revealed that there is a vesicle enveloping the heteropycnotic XY which appears to contain as much RNA as the nucleolus. The heteropycnotic bivalent was found to be situated at the pole of the nucleus opposite to that near which the idiozome (an achromatic region of the cytoplasma) lies (Fig. 7).

The primary spermatocyte of Fig. 7 has been stained with 1 per cent thionine. Vivid metachromasia of the nucleolus and the vesicle which envelops the heteropycnotic XY is demonstrated. These bodies are stained reddish purple in contrast with the normochromatic blue of the X and Y chromosomes and the chromatin network. After incubation with ribonuclease solution, the nucleolus and the vesicle of the XY become chromophobic to thionine. The metachromasia of these structures has apparently been due to the presence of RNA.

At the particular level of focus at which Fig. 7 was taken the vesicle resembles two small nucleoli attached to the terminal ends of the XY bivalent. However, by focusing up and down it was seen that the vesicle surrounds the chromosomes. Actual envelopment of the heteropycnotic XY by the vesicle may be clearly recognized on a thinner section cut at two micra (Fig. 8).

Pachytene-like stages.—The term "pachytene-like" is employed here to represent the stages following the prochromosome stage and preceding diplotene. Included are leptotene, zygotene and pachytene stages which in practice have been found relatively indistinguishable from each other in the case of the rat. There seems to be a kind of bouquet stage of short duration where the fine thread-like autosomes in process of synapsis are polarized with their ends converging toward the pole of the nucleus near which the idiozome lies. The XY bivalent is situated at the furthermost point from this pole. It is at this stage when the autosomes are in a fine thread-like condition that the heteropycnosis of the XY bivalent is most marked.

The nucleoli disappear during leptotene or zygotene and, therefore, are no longer present in what is believed to be the pachytene nucleus (Fig. 3). The vesicle enveloping the heteropycnotic XY maintains its RNA long after the nucleoli have disappeared. Nucleoli are absent from Fig. 9. However, the vesicle still shows the prominent metachromasia following staining with thionine. This same cell is pictured in Fig. 10 which shows the result obtained following digestion with RNAase. As a result of this process the vesicle has become thioninophobic.



Figs. 1 to 10 are photomicrographs and corresponding mimeographic sketches of rat primary spermatocytes at various stages of the first meiotic division. Photomicrographs were taken by Leitz Panphoto 15 × 90. *XY*, The XY bivalent; *X*, the X-chromosome; *Y*, the Y-chromosome; *A* and *B*, autosomes; *N*, nucleolus; *I*, idiozome.

Fig. 1 is a living cell at an earliest stage of the growth period by phase contrast microscopy. Fig. 2 is stained by the Feulgen method. The cell is at the second earliest stage of the growth period (prochromosome stage).

Fig. 3 (pachytene), Fig. 4 (diplotene), Fig. 5 (diakinesis) and Fig. 6 (telophase) are stained by the 1 *N* HCl–picric acid–Giemsa method [3].

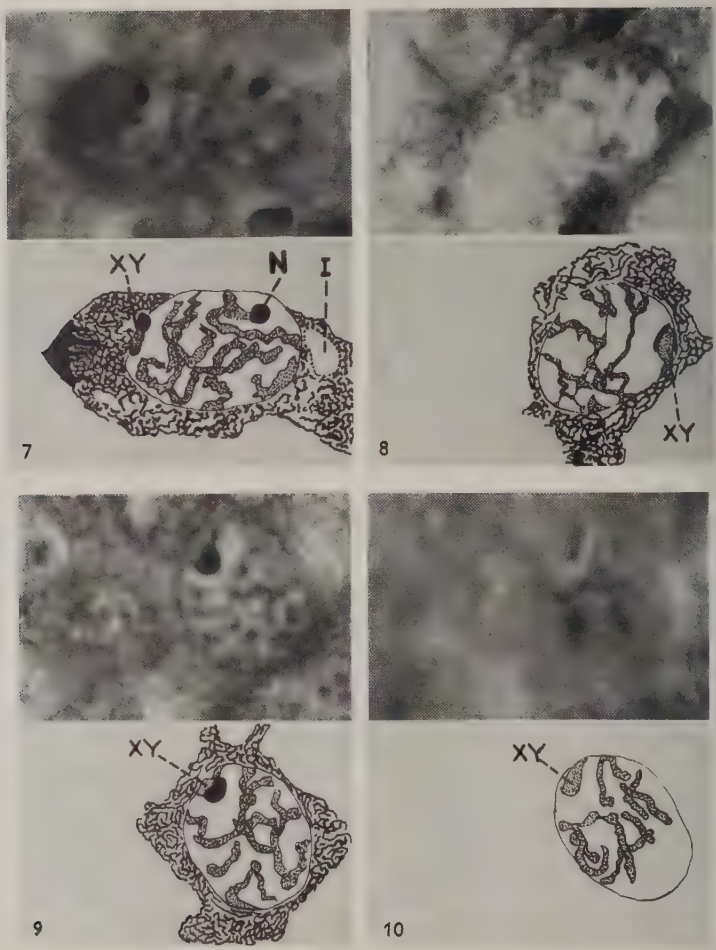


Fig. 7–10 are from paraffin sections stained with 1 per cent thionine. Excepting Fig. 8 which was cut at 2 micra, the sections were cut at 6 micra. Figs. 9 and 10 are the same nucleus stained before (Fig. 9) and after (Fig. 10) the incubation with a ribonuclease solution.

The diplotene stage.—In Fig. 4 may be recognized the typical picture of a diplotene nucleus. Homologous chromosomes have started moving apart from each other and the beginning of terminalization of chiasmata may be seen. The XY bivalent is still heteropycnotic and embedded in the vesicle

which maintains its property of metachromasia with thionine stain. There is no sign of any chiasmata between the X and the Y chromosomes.

Diakinesis and stages following.—During diakinesis, the vesicle which has been enveloping the XY bivalent finally disappears and the latter becomes isopycnotic to the autosome bivalents which are now markedly condensed and with chiasmata terminalized (Fig. 5). The large X and small Y chromosomes appear always to be joined end to end without any chiasma formation. These findings are in agreement with those of Tjio and Levan [6] but in disagreement with those of Koller and Darlington [1].

The X chromosome again becomes heteropycnotic at the telophase. Thus the daughter nuclei which have received the X chromosome are easily distinguishable from the others which have received the Y chromosome (Fig. 6). Tjio and Levan [6] state that the Y chromosome is equally heteropycnotic at the first meiotic telophase. However, in this material it has been found that the Y chromosome alone is too small to be recognized consistently, if it be heteropycnotic.

Unlike the XY bivalent, the heteropycnotic X of the first meiotic telophase is not embedded in an RNA-charged vesicle.

DISCUSSION

As early as 1928 Minouchi [2] observed and described heteropycnosis of the rat sex chromosomes. However, this behavior of the sex chromosomes in the rat seems to have escaped the notice of some of the later observers. Thus, Koller and Darlington [1] describe a normal pachytene structure of the XY pair in association with the nucleolus. This findings reported herein are in complete accord with those reported by Tjio and Levan [6]. Heteropycnotic behavior has been observed to persist from pre-leptotene through diplotene of the first meiotic prophase.

Although an association between the sex chromosomes and the nucleolus has been reported in the rat [1, 6] no such relationship was detected in the material studied for the work reported here. The vesicle which is seen to surround the sex bivalent is definitely not the nucleolus. This vesicle persists for as long as the XY bivalent remains heteropycnotic (through diplotene), while the nucleolus or nucleoli disappear as soon as the nuclear membrane loses its identity (before or during pachytene). The vesicle associated with the heteropycnotic XY shows as strong a metachromasia as that of nucleoli following staining with 1 per cent thionine. Similarly it is as pyroninophilic as nucleoli following pyronine–methyl green staining. This metachromasia

and pyroninophilia completely disappear after digestion with ribonuclease. Therefore, it is believed that the vesicle contains significant amounts of ribonucleic acid.

It seems likely that the heteropycnosis of the sex chromosomes is associated in a definite way with the RNA of the vesicle surrounding the bivalent. It would be of great interest to determine this relationship and its significance in regard to the metabolism of the maturing sex cell.

SUMMARY

1. The XY bivalent of the rat primary spermatocyte has been observed to be heteropycnotic.

2. The sex bivalent appears embedded in a vesicle which is as rich in RNA as the nucleoli. The nucleoli disappear before or during pachytene. The vesicle associated with the sex chromosomes retains its RNA and its identity through diplotene.

3. No chiasmata between the X and the Y have been observed. An end to end association, however, has been seen.

4. The heteropycnotic sex chromosomes occupy a position in the nucleus opposite the point near which lies the idiozome.

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ORIGINE CILIAIRE DES FIBRILLES SCLÉROPROTÉIQUES PÉDONCULAIRES CHEZ LES CILIÉS PÉRITRICHES

ÉTUDE AU MICROSCOPE ÉLECTRONIQUE

CH. ROUILLER, E. FAURÉ-FREMIET et M. GAUCHERY

Institut de Recherches sur le Cancer Gustave Roussy et Laboratoire d'Embryologie expérimentale du Collège de France, Paris, France

Reçu le 5 mai, 1956

L'organe fixateur des Ciliés Péritriches est essentiellement constitué par une zone ciliaire thigmotactique antapicale, la scopula [3, 5, 6, 7], au niveau de laquelle des substances protéiques, et peut-être aussi mucoprotéiques, sont élaborées. L'insolubilisation rapide de ces substances se traduit par la formation d'une plaque basale adhérente au support, puis d'un fût rigide et élastique, qui s'accroît progressivement à partir de l'aire scopulienne, et constitue la partie principale d'un pédoncule ou style.

Le pédoncule non contractile des espèces appartenant aux genres *Epistylis, Opercularia, Campanella* etc., comporte une pellicule externe formée au niveau d'un bourrelet ectoplasmique périscopulien, entourant un faisceau fibrillaire qui s'accroît à partir des cils de la scopula; les propriétés physicochimiques de ce faisceau sont comparables à celles de scléroprotéines fibreuses [10].

Chez les espèces à pédoncule contractile des genres *Intranstylum, Zoothamnium, Vorticella* et *Carchesium*, cette même structure est compliquée par la formation d'un canal central ou latéral occupé par un prolongement de la masse cytoplasmique contenant elle-même une puissante fibre myoïde, le spasmonème. Dans ces différents cas, le bourrelet périscopulien, non modifié, forme toujours la gaîne externe, mais la zone scopulienne, réduite et repoussée latéralement, donne un faisceau fibrillaire asymétrique ou hélicoïde longeant ou entourant le cordon cytoplasmique central et son myoïde. Ce faisceau, quelle que soit sa forme, fonctionne comme un système élastique antagoniste du myoïde, et provoque, après la contraction, l'extension du pédoncule jusqu'à sa longueur initiale.

L'examen au microscope électronique des structures pédonculaires [12, 21] a confirmé la nature ciliaire de la scopula; la gaine pédonculaire apparaît formée par une substance amorphe élaborée au niveau du bourrelet ectoplasmique marginal; par contre, les fibres protéiques ne résultent pas, comme

l'un de nous l'avait autrefois supposé [5, 7, 8] d'une sécrétion interciliaire, mais correspondent à l'accroissement et à la transformation directe des constituants propres du cil tels que la membrane enveloppante ou le faisceau fibrillaire. Cette solution imprévue d'un problème de morphologie comparée comporte plusieurs modalités différentes; de toute manière la notion d'une continuité entre des structures fibreuses scléroprotéiques et les constituants propres du cil est une constatation intéressante en matière de cytologie générale.

MATÉRIEL ET TECHNIQUE

Trois espèces de Ciliés Péritriches formant d'assez grandes colonies ont été utilisées; ce sont *Campanella umbellaria* Goldfuss et *Opercularia articulata* Ehrb., formes d'eau douce récoltées aux environs de Paris, et *Zoothamnium alternans* Clap. et Lachm., forme marine récoltée à Roscoff.

Les colonies des deux premières espèces ont été isolées puis fixées pendant 30 minutes avec la solution osmique de Palade tamponnée soit à pH 7.3, soit à pH 8. Les colonies de *Zoothamnium* ont été fixées pendant 15 minutes par une solution de tétroxyde d'osmium à 1 % dans l'eau de mer (pH 8), puis transférées dans la solution osmique de Palade pendant 15 minutes afin de prolonger la fixation tout en commençant l'élimination des chlorures. Après les lavages et la déshydratation progressive dans les alcools de concentration croissante, l'inclusion a été faite au *n*-butyl méthacrylate. Les sections ont été examinées au microscope R.C.A., type EMU 2E.

OBSERVATIONS

La scopula.—La structure de la scopula est aisément montrée par les techniques cytologiques courantes; les imprégnations argentiques mettent en évidence l'infraciliature; les cils sont bien visibles chez l'Infusoire vivant observé à l'éclairage ordinaire ou mieux en contraste de phase (Fig. 2).

Au microscope électronique les corpuscules ciliaires ou cinétosomes apparaissent comme des ampoules allongées, presque cylindriques ou tubulaires [14] dont la paroi est épaissie dans sa partie basale. Le centre du corpuscule tubulaire est occupé par une fibrille correspondant, semble-t-il, à la double fibrille axiale du cil. Les cils sont enrobés dans une substance de faible

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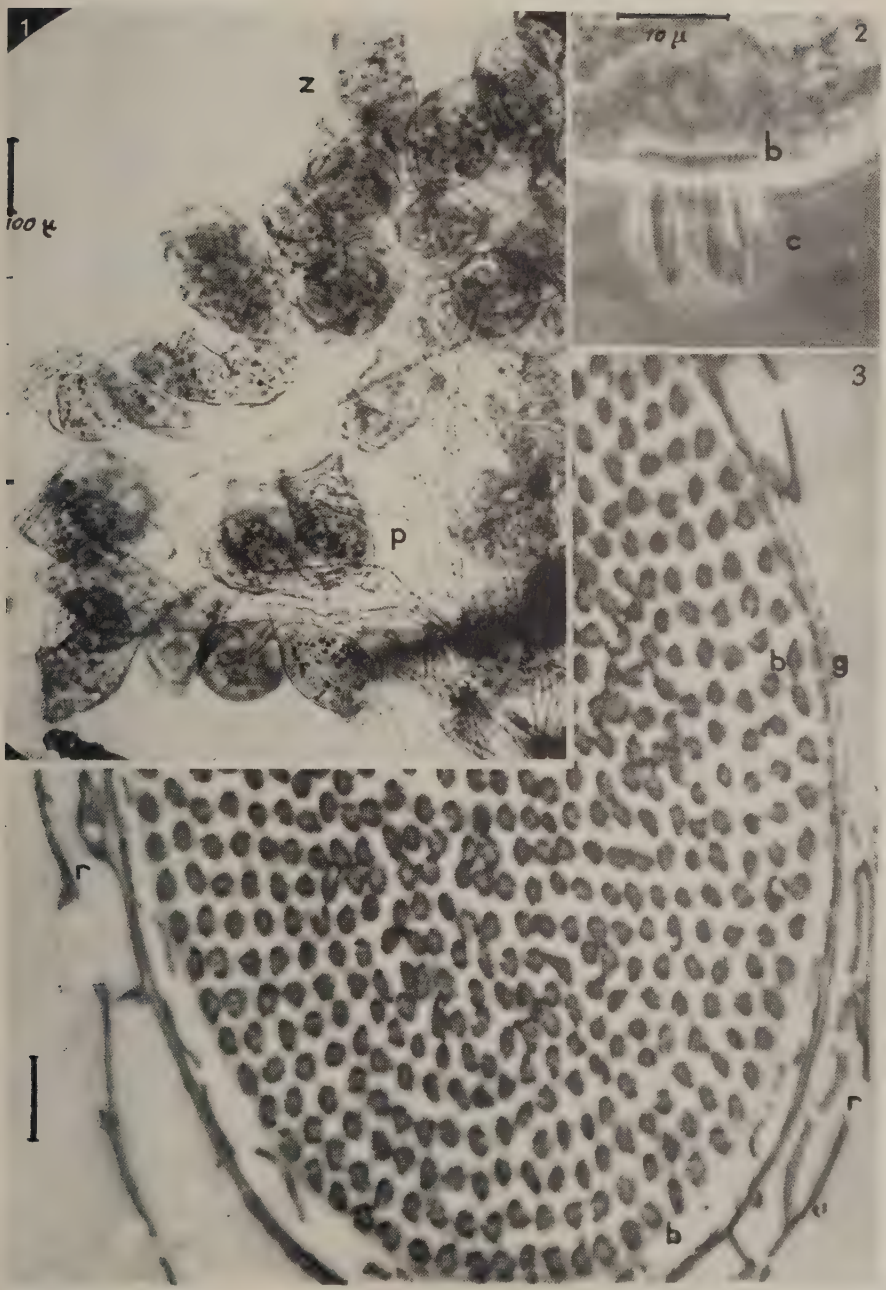
Fig. 1. *Campanella umbellaria;* microphotographie *in vivo*, d'une partie d'une colonie montrant les zoïdes *Z* portés par les branches pédonculaires grêles et flexueuses, *p.* (Trait = 100 μ.)
Fig. 2. *Campanella.* Pôle antapical d'un zoïde séparé de son pédoncule, montrant la scopula avec les corpuscules basaux *b* et les cils *c*. Microphotographie en contraste de phase. (Trait = 10 μ.)
Fig. 3. *Campanella.* Micrographie électronique d'une section parallèle au plan de la scopula et coupant les corpuscules basaux *b;* ceux qui sont régulièrement disposés en cercles concentriques portent les cils scopuliens; ceux qui sont distribués sans ordre dans la zone centrale n'en forment pas. La gaine pédonculaire *g* est entourée par des replis cuticulaires, *r.* (Trait = 1 μ).)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densité apparemment amorphe; leur structure caractéristique est marquée par la disposition circulaire des neuf fibrilles périphériques entourant la double fibrille axiale [1, 2, 16]; il est difficile de préciser si les premières commencent au niveau d'une « plaque basale », d'ailleurs peu marquée, ou sont en continuité avec la paroi du cinétosome.

Le faisceau fibreux de Campanella umbellaria.—Le pédoncule des colonies de *Campanella umbellaria* (Fig. 1) présente un fût basal épais; son diamètre diminue à chaque bifurcation correspondant à une nouvelle génération de zoïdes [8]. La masse fibreuse de ce pédoncule constitue une sorte de tube entourant un espace central vide; elle est formée elle-même par un ensemble de très fins tubules juxtaposés dans lesquels sont insérés les cils scopuliens, exceptionnellement longs, de chaque individu. Il est possible de séparer les zoïdes en déboitant les cils de la scopula qui apparaissent, à la base du corps comme une houppe de fins filaments, longs de 10 μ, nettement séparés les uns des autres quand on les examine en contraste de phase (Fig. 2). Le plateau infraciliaire rigide, est bien visible sur le Cilié vivant et les cinétosomes sont mis en évidence par l'imprégnation argentique. Les micrographies électroniques de sections parallèles au plan scopulien (Fig. 3) montrent les corpuscules ciliaires régulièrement rangés en séries concentriques autour d'une zone centrale où leur distribution est désordonnée; ils ne portent pas de cils en ce point qui correspond à l'espace central vide du pédoncule.

L'examen au microscope électronique de sections transversales du style montre un ensemble annulaire d'alvéoles juxtaposées (Fig. 6); les parois de ces alvéoles sont irrégulièrement discontinues; l'ensemble est entouré par la gaine pédonculaire, membrane apparemment homogène, épaisse de 30 mμ environ. Les sections longitudinales montrent aussitôt que les alvéoles représentent la coupe des tubules déjà indiqués par l'examen au microscope optique. Le diamètre de ces tubules est de 350 à 400 mμ; leur paroi est dessinée par un treillis de très fines fibrilles obliques et réticulées (Fig. 5), dont le diamètre est de l'ordre de 5 à 10 mμ.

L'examen de sections légèrement obliques (Fig. 4) coupant à différents

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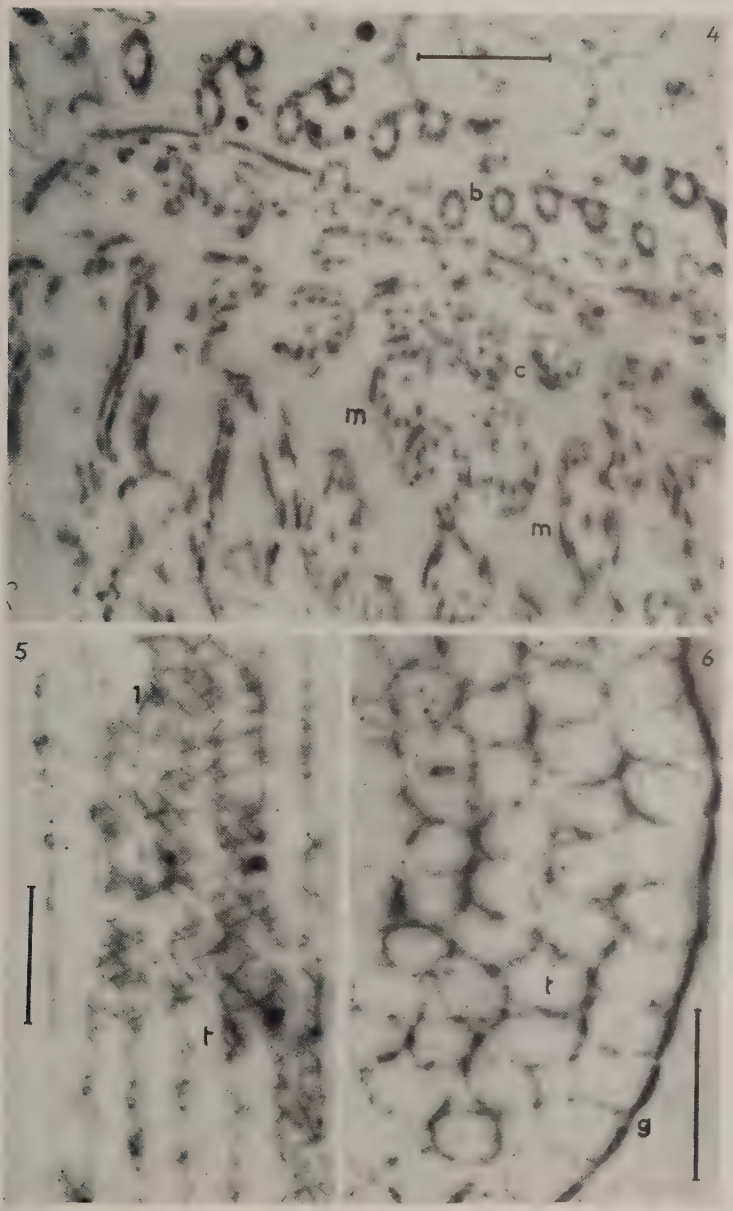
Fig. 4. *Campanella.* Micrographie électronique d'une section oblique de la scopula; les corpuscules basaux *b* sectionnés tout près de l'origine des cils, montrent les neuf fibrilles périphériques mais non la double fibrille centrale; celle-ci apparaît un peu plus haut, lorsque les cils proprement dits *c* commencent à s'élargir; la membrane ciliaire *m* présente un aspect fibreux. (Trait = 1 μ)
Fig. 5 *Campanella.* Micrographie électronique d'une section longitudinale du pédoncule montrant le lacis de fines fibrilles *l* qui délimite les espaces tubulaires *t*. (Trait = 1 μ.)
Fig. 6. *Campanella.* Micrographie électronique d'une section transversale du pédoncule montrant les espaces tubulaires *t* juxtaposés délimités par le lacis fibrillaire et la gaine externe *g*. (Trait = 1 μ.)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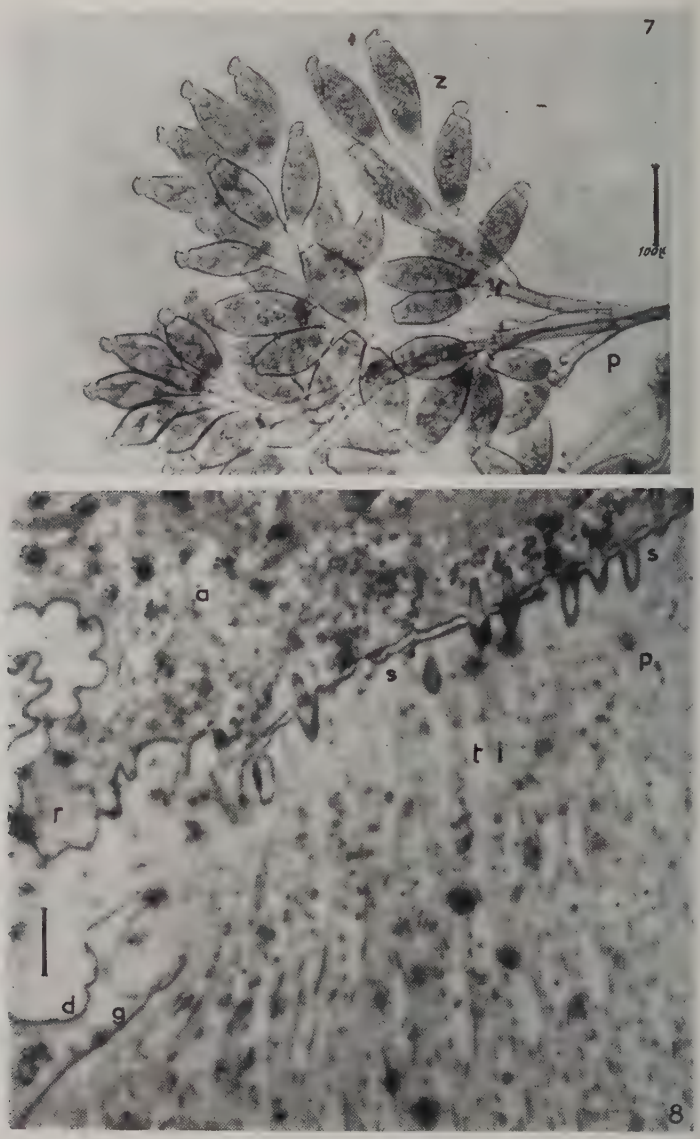
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niveaux les cils de la scopula est nécessaire pour interpréter ces structures; partant de la région apicale des cinétosomes qui montrent déjà les neuf fibrilles ciliaires périphériques, on voit les cils proprement dits s'élargir rapidement jusqu'à venir au contact les uns des autres. Leur membrane propre paraît alors relativement épaisse; sa structure semble discontinue, et, un peu plus loin, elle se résout en fibrilles irrégulièrement enchevêtrées dessinant la paroi des espaces tubulaires qui dépassent maintenant la longueur propre du cil, de sorte que le faisceau des fibrilles périphériques et axiales en est absent.

Il apparaît ainsi que, chez *Campanella*, la structure fibreuse du pédoncule n'est autre chose que le prolongement des membranes ciliaires, probablement reliées par une substance amorphe interstitielle formée entre les cils eux-mêmes, au contact de la surface scopulienne. Il est probable que ce type structural se retrouve chez d'autres espèces du genre *Epistylis*.

Le faisceau fibreux d'Opercularia articulata. L'aspect fibreux massif du pédoncule d'*Opercularia* [3, 4, 5] traduit une structure canaliculaire (Fig. 7) comparable, au premier abord, avec celle observée chez *Campanella* (Fig. 8). Les sections transversales montrent la coupe de ces canalicules comme autant d'alvéoles circulaires serrées les unes contre les autres, séparées par une substance interstitielle amorphe. Mais une différence significative entre ce type de structure et le précédent est indiquée par la présence constante autour de chaque espace canaliculaire (Fig. 10), de neuf fibres longitudinales régulièrement espacées; ce simple fait apparaît déjà comme la marque évidente d'une structure ciliaire.

Les sections longitudinales (Fig. 11) montrent, d'autre part, que chacune de ces fibres longitudinales, épaisse de 50 mμ environ, présente une structure périodique caractérisée par l'alternance régulière de bandes transversales sombres et claires; la période est de l'ordre de 440 Å.

Les sections intéressant la scopula (Fig. 8 et 9) montrent ici encore les corpuscules ciliaires sous la forme d'éléments tubulaires longs de 0.5 μ environ, épaissis vers la base et dans lesquels les fibrilles ciliaires semblent prendre naissance. Entre les bases des cils, la pellicule épiplasmique paraît être doublée par une pellicule superficielle au dessus de laquelle se trouve la

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Fig. 7. *Opercularia articulata;* microphotographie *in vivo* d'une partie d'une colonie montrant les zoïdes *Z* portés par les branches pédonculaires rigides, *p*. (Trait = 100 μ.)

Fig. 8. *Opercularia.* Micrographie électronique d'une section presque longitudinale passant par la scopula, *s* et le pédoncule, *p* ; *a* : cytoplasme antapical et ses différenciations myoïdes; *r* : repli ectoplasmique périscopulien avec les crêtes sous cuticulaires, et la cuticule *d*, décollée par les réactifs; *g* : gaine du pédoncule ; *t* : espaces tubulaires longitudinaux entourés par la substance interstitielle *i*. (Trait = 1 μ.)

substance interstitielle amorphe contenant quelques granulations irrégulièrement dispersées.

La partie suprabasale d'un cil apparaît comme une massue de structure assez dense, haute de près de 1 μ, autour de laquelle les neuf fibrilles ciliaires périphériques s'écartent progressivement les unes des autres en même temps qu'elles s'élargissent; elles se continuent plus loin tout au long de la paroi interne de chacun des canalicules dont l'assemblage à travers la substance interstitielle amorphe, réalise la structure caractéristique du pédoncule.

Lorsque les fibrilles commencent à s'écarter de la courte massue ciliaire, elles accusent déjà une très nette striation transversale (Fig. 9): mais la périodicité n'est, ici, que de 220 Å environ, et c'est à quelque distance du lieu de formation de ces fibrilles (Fig. 11) que leur structure définitive est atteinte avec une périodicité proche de 440 Å.

Nous discuterons plus loin la signification possible des structures périodiques observées. Le fait sur lequel nous insisterons présentement est que la structure fibreuse du pédoncule d'*Opercularia* correspond à la prolongation, par un accroissement apparemment continu, des fibrilles ciliaires périphériques.

Le faisceau fibreux de Zoothamnium alternans.—Zoothamnium alternans (Fig. 12 et 13) est un Péritriche colonial marin dont le pédoncule est contractile. Le zoïde migrateur qui se fixe par sa scopula pour fonder une colonie [9] forme d'abord un fut pédonculaire massif et rigide, de structure fibreuse; lorsque ce fût basal atteint une certaine hauteur, la scopula est repoussée latéralement, et, tandis que la gaine externe continue de s'accroître sous sa forme initiale, le faisceau fibreux s'allonge latéralement sur le coté d'un prolongement du corps du zoïde dont la base reste fixée à la partie du fût qui a cessé de croître. Une section transversale montre donc, à l'intérieur de la gaine externe, le faisceau fibreux d'un côté et le cordon cytoplasmique avec sa différenciation myoïde, ou spasmonème, de l'autre (Fig. 14).

Au niveau de la zone scopulienne les corpuscules basaux des cils apparaissent, sur les micrographies électroniques, avec le même aspect tubulaire

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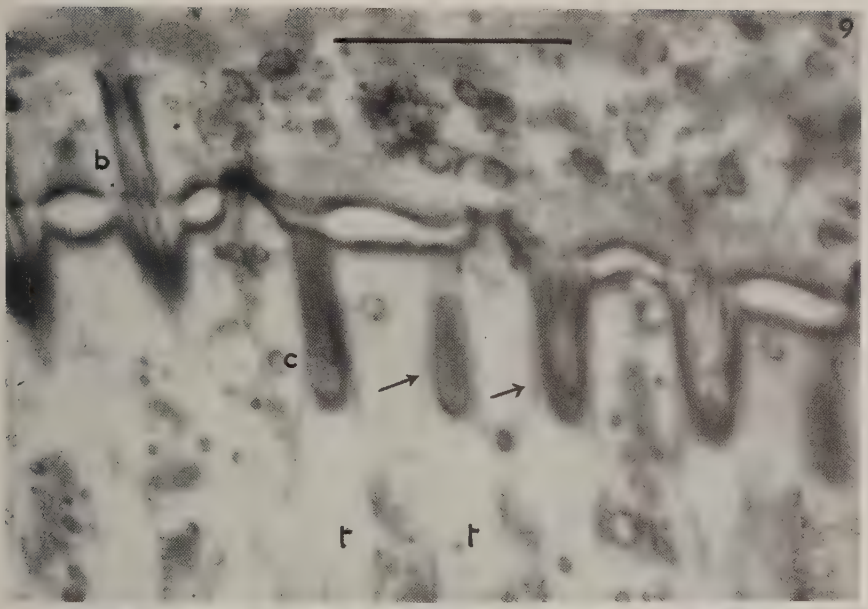
Fig. 9. *Opercularia.* Micrographie électronique d'une section de la scopula; *b* : corpuscule basal avec l'origine des fibrilles ciliaires; *c* : prolongement ciliaire central en forme de massue; les fibrilles périphériques s'en écartent et montrent une striation transversale à courte période (indiquée par la flèche); *t* : espaces tubulaires. (Trait = 1 μ.)

Fig. 10. *Opercularia.* Micrographie électronique d'une section transversale du pédoncule; *t* : coupe des espaces tubulaires limités par les 9 fibrilles ciliaires périphériques *b*; *i* : substance interstitielle; *g* : gaine pédonculaire. (Trait = 1 μ.)

Fig. 11. *Opercularia.* Section longitudinale du pédoncule montrant les fibrilles ciliaires, *f* avec leur structure périodique. (Trait = 1 μ.)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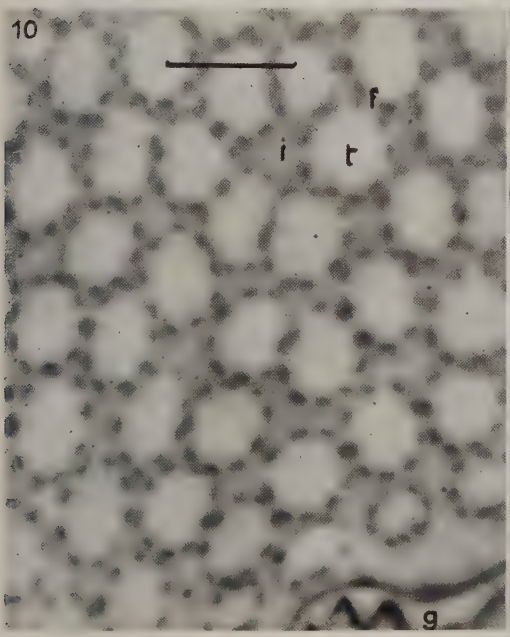
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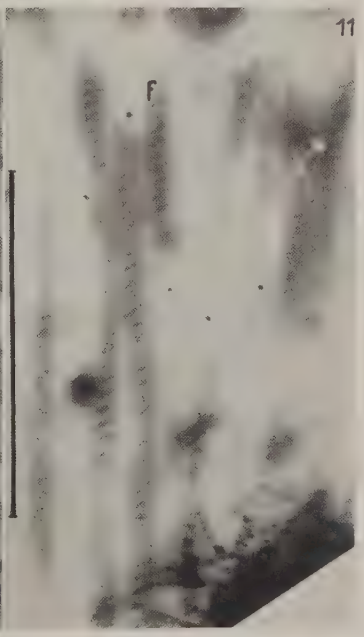
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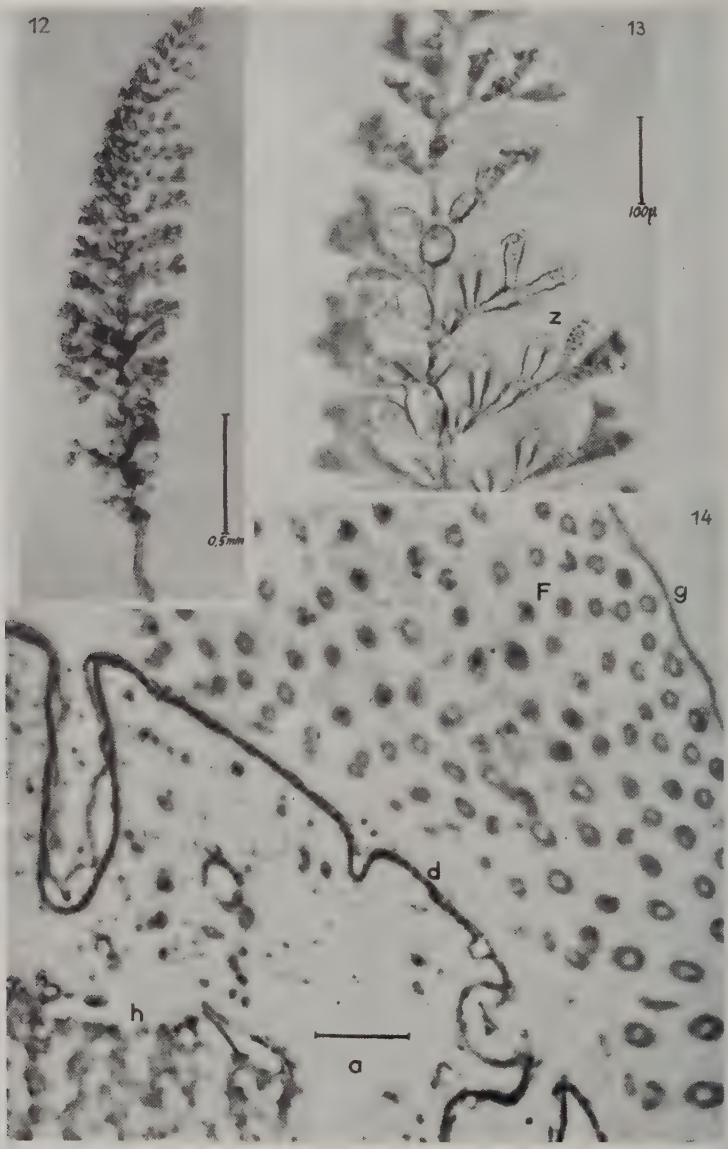
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décrit chez les espèces précédentes (Fig. 15). Les cils sont nettement dessinés par leur faisceau fibrillaire dont le diamètre mesure près de 350 Å, mais leur membrane n'est pas visible, ou fait défaut de sorte que les neuf fibrilles périphériques semblent directement entourées par la substance amorphe interstitielle.

La structure caractéristique des cils occupe une longueur de 1 μ environ, au delà de laquelle les fibrilles périphériques se rapprochent les unes des autres jusqu'à devenir jointives et à former la paroi d'un tube dont le diamètre extérieur mesure environ 200 mμ. Ce sont ces prolongements ciliaires qui, baignés par la substance interstitielle, forment le faisceau fibreux élastique du pédoncule.

Comme chez *Opercularia*, les fibrilles ciliaires périphériques montrent, chez *Zoothamnium*, une structure périodique, marquée par la même alternance de bandes transversales sombres et claires, et cette structure est particulièrement évidente sur la paroi des fibres tubulaires qui prolongent chaque cil; la lumière du tube est occupée par une fibrille axiale dont la structure paraît être continue. La périodicité de la striation transversale accuse d'abord, lorsque les fibrilles ciliaires se rapprochent, une valeur faible, voisine de 280 Å avant d'atteindre une valeur apparemment constante de 470 Å environ tout au long de la fibre tubulaire.

DISCUSSION

Le pédoncule fixateur des Ciliés Péritriches comporte d'une part deux sortes de structures rigides et élastiques : la gaine externe et le faisceau fibreux; et de l'autre une substance interstitielle amorphe. Chez les formes contractiles, une fibre myoïde contenue dans un cordon cytoplasmique, se surajoute à ces trois constituants.

La gaine externe et la substance interstitielle sont formées à la surface du corps, la première au contact d'un bourrelet marginal, la seconde au contact de la surface scopulienne, entre les cils. L'une et l'autre représentent des produits d'élaboration ou de sécrétion, comparables dans une large mesure

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Fig. 12. *Zoothamnium alternans*. Ensemble d'une colonie photographiée *in vivo* en extension. (Trait = 0,5 mm.)

Fig. 13. *Zoothamnium*. Détail d'une colonie photographiée *in vivo*, montrant l'axe principal et quelques rameaux avec les zoïdes *Z*; l'aisselle de trois rameaux porte les zoïdes migrateurs en voie d'accroissement. (Trait = 100 μ.)

Fig. 14. *Zoothamnium*. Micrographie électronique d'une section transversale du tronc pédonculaire. *g* : gaine externe; *F* : fibres d'origine ciliaire; *a* : prolongement cytoplasmique central limité par la pellicule, *d* et contenant la fibre myoïde, *h*. (Trait = 1 μ.)

à ceux qui constituent la thèque des Vaginicolidae et qui sont élaborés à la surface basale du corps et au niveau de la collerette de ces Péritriches [4].

Le faisceau fibreux, qui constitue généralement la structure élastique la plus importante et la plus caractéristique du pédoncule, est formé par un ensemble de fibres ou de canalicules qui sont en continuité avec les cils de la scopula. Ces fibres ou ces canalicules ne sont autre chose que le prolongement de structures proprement ciliaires, telles que la membrane du cil ou ses neuf fibrilles périphériques.

Il apparaît ainsi que le faisceau fibreux d'une part, la gaine périphérique et la substance interstitielle de l'autre, ont une origine différente; on sait d'ailleurs, que, chez quelques espèces, ces deux constituants peuvent accuser de remarquables allométries de croissance [11].

La nature ciliaire des constituants fibreux du pédoncule des Péritriches est intéressante à l'égard de la morphologie comparée des Ciliés; dans un autre groupe, celui des Chonotriches, les *Chilodochona* forment un pédoncule rigide et élastique qui ressemble, à première vue, à celui d'un *Epistylis;* mais dans ce cas les fibres pédonculaires protéiques sont sécrétées par autant de glandules cytoplasmiques [15] comparables à celles qui secrètent la substance adhésive du pied des Dystériens. Ces deux sortes de pédoncules ne sont donc pas cytologiquement comparables.

Les réactions microchimiques et l'action des ferments protéolytiques montrent que les constituants élastiques du pédoncule des Péritriches sont principalement constitués par des protéines insolubles [10]. Leurs propriétés physiques : haute élasticité, contraction thermique, anisotropie optique etc., sont celles des scléroprotéines en général. La présence de composés polysaccharidiques n'a pas été démontrée, mais elle n'est pas exclue cependant.

La structure périodique reconnue chez les fibres et les fibrilles pédonculaires d'*Opercularia* et de *Zoothamnium*, et constatée également chez celles de *Carchesium* par Randall [20], rappelle la structure des fibrilles collagènes; mesurées au niveau des cils scopuliens les périodes trouvées, soit 220 et 280 Å sont comparables à celle des fibrilles collagènes jeunes [17, 18, 19]; mais les valeurs de 440 et 470 Å montrées par les fibres et les fibrilles définitives sont inférieures à celle (640 Å) qui caractérise les collagènes adultes et se

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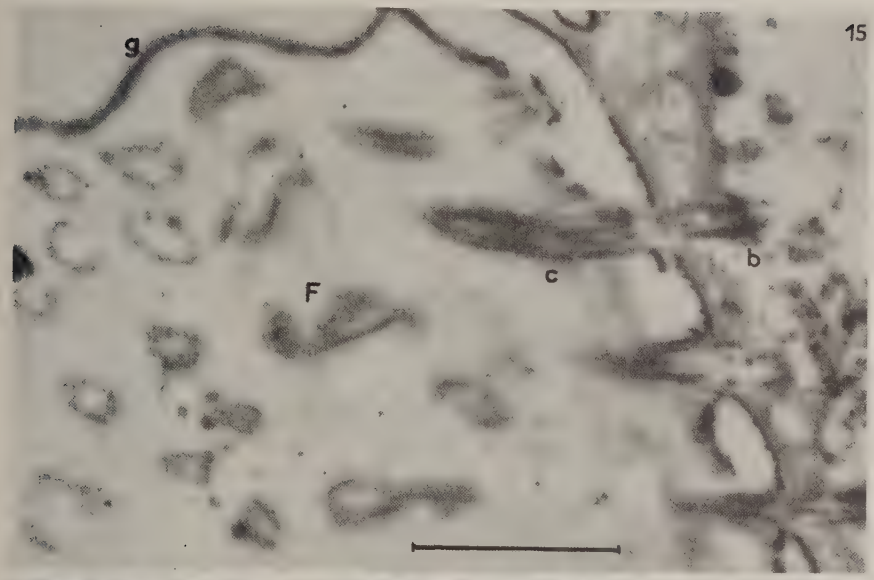
Fig. 15. *Zoothamnium*. Micrographie électronique d'une section de la scopula. *b* : corpuscule basal; *c* : cil scopulien; *F* : fibres prolongeant les cils et montrant une striation transversale à périodicité courte. (Trait = 1 μ.)

Fig. 16. *Zoothamnium*. Micrographie électronique d'une section parallèle aux fibres tubulaires, *F* formées par la coalescence des fibrilles ciliaires périphériques ; ces fibres montrent la structure périodique définitive; la fibrille centrale, *f* est apparemment homogène (Trait = 1 μ.)


g
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c
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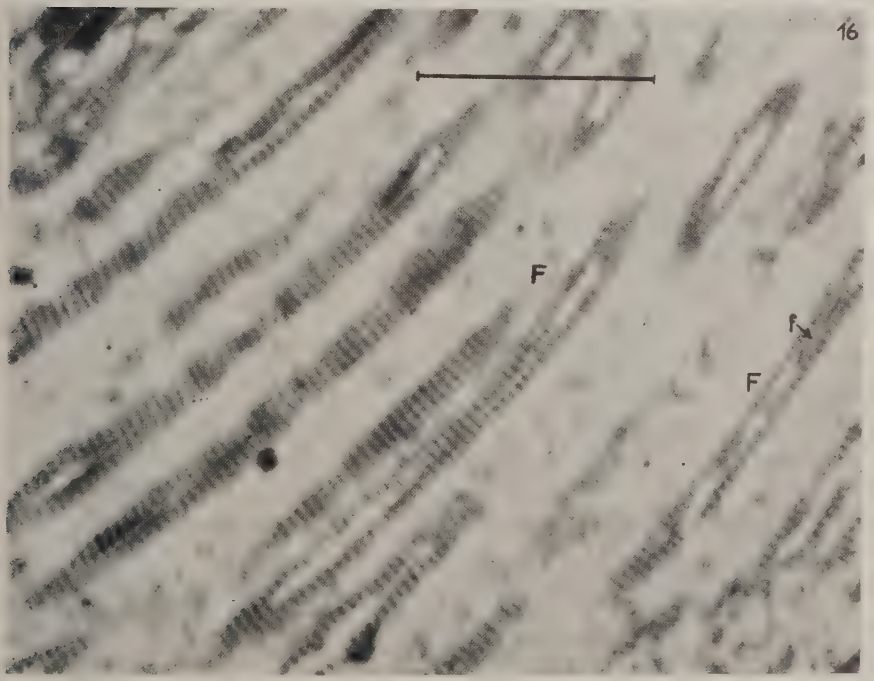
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rapprochent davantage de celle montrée par différents trichocystes, soit 500 Å. On remarquera d'autre part qu'il n'existe pas, comme chez les collagènes proprement dits, de striations intermédiaires entre les stries transversales principales.

La structure périodique paraît être propre aux fibres pédonculaires formées par l'accroissement des fibrilles périphériques des cils scopuliens (*Opercularia, Zoothamnium, Carchesium*). On ne la constate pas chez les constituants du lacis fibrillaire issus de la membrane propre de ces cils (*Campanella*). C'est que probablement, la membrane et les fibrilles ciliaires diffèrent quelque peu quant à leurs constituants chimiques [12], la présence de polysaccharides dans la membrane étant vraisemblable.

La structure et la morphogenèse du pédoncule fixateur des Ciliés Péritriches doit être considérée comme un cas très particulier de différenciation cellulaire et d'adaptation fonctionnelle. Mais ce cas pose un intéressant problème de cytologie générale, en montrant la participation directe d'un organite ciliaire à l'édification de fibres scléroprotéiques, par l'accroissement continue de ses propres constituants.

A l'égard de la morphologie comparée des Ciliés, il est remarquable que cette adaptation morphogénétique, caractérisée par l'utilisation si particulière du cil et de ses constituants, soit propre à un groupe bien défini,[1] et que, dans ce même groupe, cette utilisation comporte plusieurs modalités différentes.

RÉSUMÉ

La partie élastique du pédoncule fixateur des Ciliés Péritriches est principalement constituée par un faisceau fibreux de nature scléroprotéique.

Les structure fibreuses constituant ce faisceau sont en continuité avec les cils de la scopula et sont formées par l'accroissement continu de leurs constituants.

Trois modalités différentes ont été reconnues; chez *Campanella*, la membrane ciliaire se prolonge en un lacis fibrillaire délimitant un espace canaliculaire; chez *Opercularia* et *Zoothamnium*, les neuf fibrilles périphériques du cil s'accroissent et acquièrent un structure périodique qui évoque celle des fibrilles collagènes. Mais, tandis que chez *Opercularia* ces neuf fibrilles s'écartent les unes des autres, elles s'accolent étroitement chez *Zoothamnium* de manière à constituer une fibre tubulaire apparemment simple.

[1] Les Ciliés tentaculifères forment un pédoncule fixateur qui ressemble à celui des Péritriches; nous n'avons pas retrouvé cependant une structure comparable et de nouvelles recherches doivent être poursuivies.

SUMMARY

The main structure of the peritrichous stalk is a flexible bundle of scleroproteic fibrils. These fibrils are continuous with the cilia of the scopula and result in the continued growth of either the ciliary membrane *(Campanella)* or of the nine ciliary fibrils *(Opercularia, Zoothamnium)* which exhibit periodic striation called collagenous fibrils.

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THE ULTRASTRUCTURE OF MOUSE LUNG FINE STRUCTURE OF THE CAPILLARY ENDOTHELIUM[1]

H. E. KARRER

Johns Hopkins University, School of Hygiene and Public Health, Department of Pathobiology, Baltimore, Md., U.S.A.

Received May 20, 1956

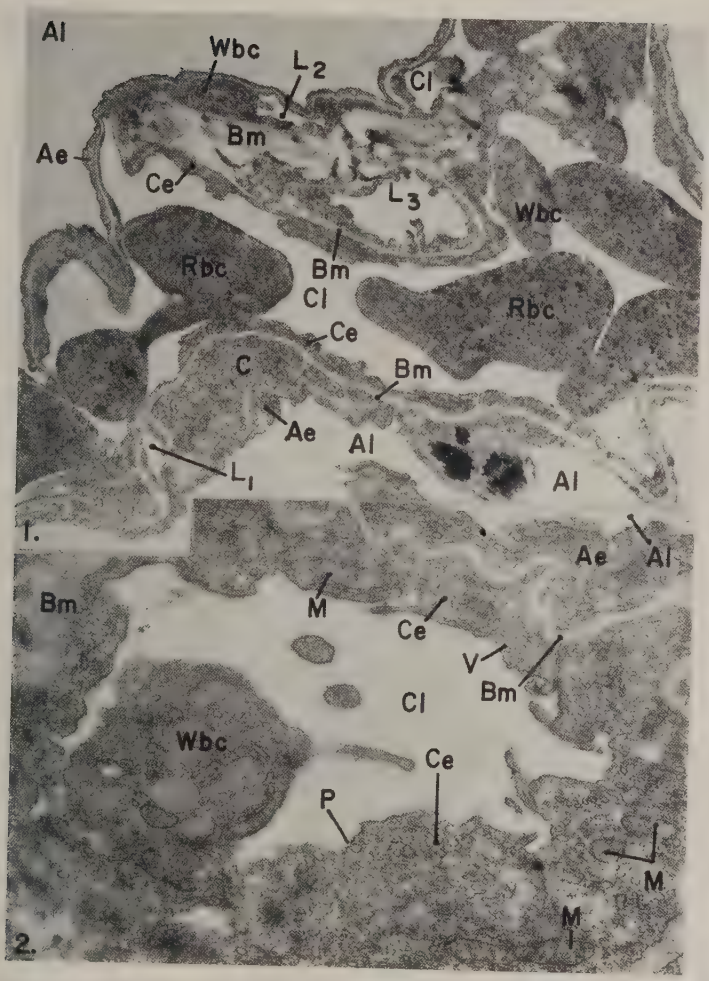
The fine structure of capillary endothelium, as seen in the electron microscope, has not been studied extensively up to the present. Palade [7] stressed the occurrence of numerous small vesicles inside endothelial cells, and some findings in endothelium made incidental to the study of alveolar walls have been reported [3, 4]. The present paper presents a few additional findings on the fine structure of the thin portions of the endothelium.

The tissue processing methods that were used have already been described in their essential points [3, 4]. The tissue depicted in Figs. 1, 2 and 7 was treated with ammonium sulfide solution [4]. A Servall microtome [9] and glass knives were used for microtomy, and an RCA EMU 2 electron microscope for microscopy.

The lung capillaries inside the inter-alveolar septa are seen to be lined by an uninterrupted endothelial layer, which is separated from the alveolar epithelium by the basement membrane [3, 4] (Figs. 1, 2). In some places the basement membrane is seen to divide into two layers which enclose parts of cells, or lumina that are lined by cells (Fig. 1). Some of these lumina are recognizable as belonging to a capillary, whereas others are lined by alveolar epithelium and therefore represent alveolar air space (Fig. 1).

In the cytoplasm of alveolar epithelial cells numerous small (about 30–45 mμ) round or oblong vesicles [7] are seen, which are most obvious in micrographs of low magnification (Fig. 2). Similar structures are also seen inside the alveolar epithelium (Fig. 2). These vesicles are lined by a dense membrane. The fact that some of them apparently are invaginations of the plasma membrane of the endothelial cell (Figs. 2, 3) suggests that they possibly arise at the inner cell surface and are the product of pinocytosis by the cell [3, 4, 7). Parts of cells that are enclosed between two layers of basement membrane also contain similar structures (Fig. 1), and may represent tan-

[1] Supported in part by a Grant-in-Aid of the National Cancer Institute, United States Public Health Service.



Abbreviations.—*Ae* alveolar epithelium; *Al* alveolar lumen; *Bm* basement membrane; *C* part of cell between layers of basement membrane; *Ca* cutting artefact; *Cb* boundary between endothelial cells; *Ce* capillary endothelium; *Cl* capillary lumen; *L* lumen; *M* mitochondrium; *Mg* mitochondrial granule; *P* pinocytosis; *Rbc* red blood cell; *V* vesicle; *Wbc* white blood cell.

Fig. 1. Capillary inside an interalveolar septum. One small lumen (L_1) can be identified as belonging to an alveolus, another one (L_2) as being part of the capillary. A third one (L_3) is unidentified. Cellular material enclosed by the basement membrane (*C*) could belong either to the endothelium, or to the epithelium. × 10,240.

Fig. 2. Rather thick portion of capillary endothelium, showing vesicles (*V*) and mitochondria (*M*). Part of the mitochondrium towards the upper margin of the figure is shown in Fig. 7. × 27,200.

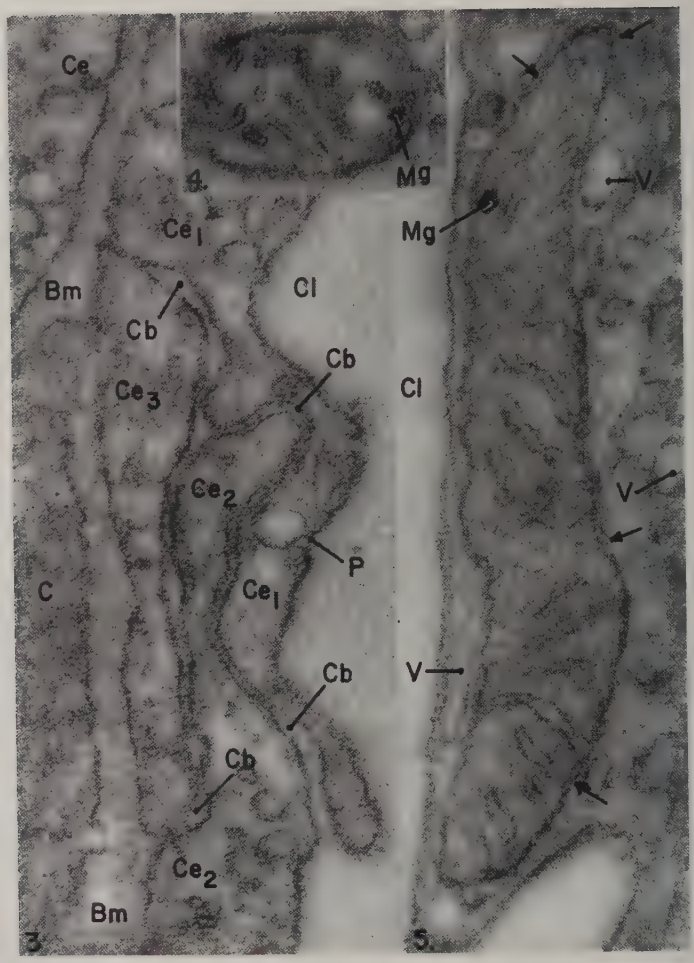


Fig. 3. Intercellular boundary between endothelial cells (Ce_1, Ce_2). The overlap of the cells and the absence of a "terminal bar" is noted. Ce_3 may belong either to a third cell, or represent a portion of either Ce_1 or Ce_2. The cell enclosed by the basement membrane (C) is not identified. × 88,800.

Fig. 4. Mitochondrium of endothelial cell, demonstrating the tubular nature of the internal structures. × 93,600.

Fig. 5. Mitochondrium, showing the internal structures which here resemble "cristae". The doubleness of the outer membrane is evident in places (arrows). × 113,600.

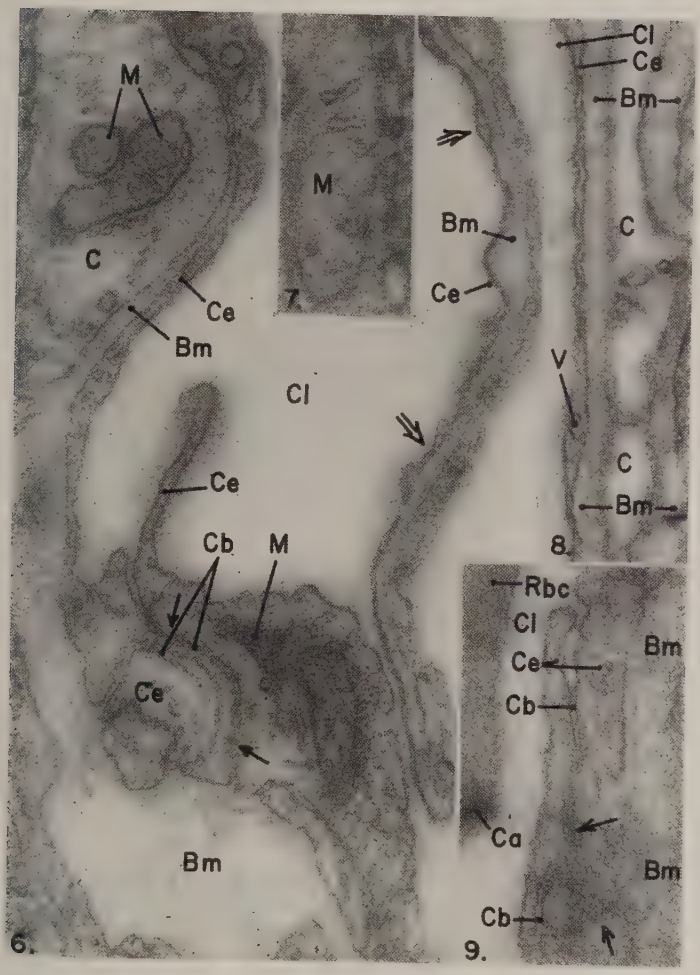


Fig. 6. The illusion of a "terminal bar" is created (arrows) in two slightly obliquely sectioned portions of an intercellular boundary between endothelial cells. The tubular nature of the mitochondrial inner structures is recognized within the mitochondrium in the lower half of the figure. The two plasma membranes of the thin endothelial layer at right are resolved only in places (double arrows). × 45,600.

Fig. 7. Part of a mitochondrium, showing the tubular inner structures. × 108,000.

Fig. 8. Very thin portion of an endothelial cell. A part of a cell enclosed by basement membrane (*C*) is unidentified. × 80,800.

Fig. 9. Intercellular boundary between two endothelial cells, with the illusion of "terminal bars" (arrows) created by oblique sectioning, and possibly, by cutting artefact. × 54,400.

gentially sectioned portions of either endothelium or of epithelium. Even the very thinnest portions of the endothelium may contain vesicles (Fig. 8). The number of vesicles varies in different capillaries, and may be found considerably less than in Fig. 2. No dense particles [8] are seen adhering to the membrane that limits the vesicles, although they do occur in relation to short fragments of elongated, apparently tubular endoplasmic reticulum inside capillary endothelial cells (not shown in the presented micrographs).

The mitochondria of endothelial cells are filamentous, and in sections appear as oblong or as round profiles (Figs. 2, 4, 5, 6, 7). It is believed that their outer membrane is double, and that the two components of this membrane are so closely approximated (3.5 mμ, where measurable) that they can be distinguished only in portions of rather highly magnified images of mitochondria (Fig. 5). In other micrographs the mitochondrial membrane cannot be recognized as double (Figs. 4, 6, 7), probably due to either oblique sectioning or inadequate resolution. The mitochondrial matrix [5, 6] shows dense granules [5, 6] as well as structures which in some sections resemble mitochondrial cristae [5, 6] or inner double membranes [10, 12, 13, 14, 15] (Fig. 5), but which in other sections are recognized to be tubular (Figs. 4, 6, 7), thus resembling the tubules found inside mitochondria of other species and cells [1, 2, 11].

Where two endothelial cells lie together they frequently overlap (Figs. 3, 6, 9), and sometimes a third cell is seen in connection with such a cell boundary (Figs. 3, 6). In micrographs where the intercellular boundary is sectioned transversely the plasma membranes of the adjoining cells are distinct, and no "terminal bars" are seen (Fig. 3). Where the boundary is sectioned obliquely (Figs. 6, 9) the illusion of a "terminal bar", i.e. a blurring and increased density of the intercellular boundary, may be created.

The extreme thinness of portions of the endothelium may, especially under slightly oblique sectioning, create the illusion of endothelial defects in these portions (Fig. 6), but in suitable micrographs the two uninterrupted plasma membranes of the thin (10 mμ) endothelial layer can usually be demonstrated (Fig. 8).

SUMMARY

The endothelium of mouse lung capillaries has been studied with the electron microscope. The endothelium is continuous even in its thin portions. It contains in its cytoplasm numerous small vesicles, endoplasmic reticulum with adhering dense particles, and filamentous mitochondria. At least some

of the inner mitochondrial structures are tubular rather than lamelliform. The plasma membranes of adjoining endothelial cells are distinct, and no "terminal bars" can be recognized with certainty.

Thankful acknowledgement is given to Dr. D. A. Cameron for his help during the preparation of the manuscript.

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MITOCHONDRIAL POPULATIONS DURING DEVELOPMENT OF THE SEA URCHIN

J. R. SHAVER[1]

Division of Biology, Kerckhoff Laboratories, California Institute of Technology, Pasadena, Calif., U.S.A.

Received May 20, 1956

The role of cytoplasmic particles has been emphasized in several theories of embryonic differentiation [2, 5, 10], but there is little information on the numbers of these particles in the cells of the developing embryo. The majority of the earlier studies on changes in number of mitochondria during embryonic development, summarized in [17], were based on observations of fixed, stained material. Several investigators reported a decrease in the number of mitochondria per cell during cleavage and early differentiation. This decrease was thought by some to be correlated with an increase in the numbers of secretory granules, plastids, sarcosomes, etc., which were presumed to have had their origin from mitochondria.

The most recent studies of mitochondria in a developing system are those of Gustafson and his collaborators on the sea urchin [6, 7, 11]. It is stated by Gustafson and Lenicque [7] that the number of mitochondria in early cleavage stages is low, rises somewhat in the early blastula, and rises sharply again at the mesenchyme blastula stage. According to these investigators [6], this peak is followed by a decrease in numbers of mitochondria in subsequent stages.

One of the features of Gustafson's concept of sea urchin differentiation [5] is the pattern of the cells in mesenchyme blastulae and gastrulae with reference to their mitochondrial content. He states [5] that a gradient of mitochondria can be seen, decreasing from the animal pole. The evidence presented for the existence of this mitochondrial gradient [6] was obtained from counts of the particles made on intact, vitally stained embryos. Shaver [14], using similar methods, has failed to find such a pattern of mitochondrial distribution in comparable stages of two sea urchin species, and has discussed [15] the significance of these results.

Since the statements [7] concerning the overall increase and decrease of mitochondria during early development of the sea urchin are based on the

[1] Public Health Service Special Research Fellow of the National Cancer Institute. Present address: Department of Zoology, Michigan State University, East Lansing, Michigan, U.S.A.

same cytological technique with which the question of the mitochondrial gradient was studied, it seemed appropriate to attempt an enumeration of mitochondria in sea urchin embryos in another way. Accordingly, a method described by Shelton, Schneider and Striebich [16] for counting mitochondria in homogenates of mouse liver, was adapted for use with sea urchin developmental stages. A brief summary of the results of this study is included in reference [15].

MATERIAL AND METHODS

Eggs and embryos of two species of sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*, were studied. Embryos of the former species were cultured at a constant temperature of 17°C; those of the latter at an average temperature of 19.5°C. Pre-hatching stages were obtained from eggs which had been demembranated before fertilization with trypsin (0.01 per cent in sea water for ten minutes, followed by thorough washing), or, after fertilization, by drawing the eggs through a 20-gauge hypodermic canula into a syringe. Aliquots for embryo counts were removed from thoroughly mixed suspensions with a large orifice pipette. Two samples from the same suspension were counted in all cases. The volumes of the suspensions and homogenates, and of the aliquots removed, as well as the dilutions employed in making all the embryo and mitochondrial counts reported, are given in the statistical section. To insure minimal loss of material, embryos, after counting and concentration by light centrifugation, were transferred to the homogenization vessel in 10 ccm^3 portions of the sucrose medium. The homogenization vessel was a 50 ccm^3 glass serum bottle with a self-sealing rubber cap, through which a 20-gauge hypodermic canula attached to a 10 ccm^3 syringe was inserted. The bottle was attached, inverted, to an automatic pipetting device, which was connected through an electrical timer, so that the number of excursions of the syringe plunger, of equal amplitude, was the same in each homogenization. All operations, except for the counting of mitochondria, were carried out at 4°C. Aliquots of the homogenates were examined microscopically to ascertain that all embryos had been homogenized. Four homogenates of each stage studied were prepared and counted in the same experiment (with three exceptions; see Fig. 5). In almost all cases, two homogenates were made from eggs of one female, the other two from the eggs of another. The total number of homogenates sampled for counting mitochondria for each age of the two species is indicated in Figs. 5 and 6.

The Petroff-Hauser bacterial counting chamber employed in counting mitochondria was always filled in the same manner. All counts were made using a dark M oil immersion phase contrast objective (97×; 1.25 n.a.), with corresponding phase plate, and a 15 × ocular.

RESULTS

Some results of preliminary studies made to ascertain optimal conditions for the homogenization of the sea urchin stages, and for the identification

TABLE I

Effect of osmotic concentration of medium on mitochondrial structure.
L. pictus, unfertilized eggs, homogenized in buffered 1.1 *M* sucrose.

Homog. dil. 1:9 to molarity	No. of "normal" mitochondria per 0.001 mm³	Appearance of particles
1.10	32	"Normal" mitochondria
0.65	19	Fading; clumping
0.33	2	Increase in small particles
0.11	2	Increase in small, spherical refractile bodies

and counting of the mitochondria may first be given. When mitochondria were released by homogenization into 1.1 *M* sucrose, they appeared more normal than in other media (sea water, isotonic solutions of KCl, NaCl, and 1:1 mixtures of these solutions with 1.1 *M* sucrose). The sensitivity of mitochondria from sea urchin embryonic cells to changes in osmotic concentration is illustrated in Table I. The clumping of the mitochondria in media of tonicity lower than 0.88 molar, was striking. A similar phenomenon has been reported [16] for mitochondria of mammalian liver cells.

A phosphate buffer (Sörensen's) at pH 7.4–7.6 was used in making up the sucrose medium. It was evident that the appearance and shape changes

TABLE II

Effect of pH of medium on mitochondrial structure.
L. pictus, 26 hrs. (17°C), homogenized in 1.1 *M* sucrose, buffered. pH varied with 0.1 *N* HCl or NaOH.

Sample No.	pH	No. of "normal" mitochondria per 0.001 mm³	Appearance of particles
1	7.45	80	"Normal" mitochondria
2	6.50	30	Fading; clumping
3	3.22	27	Clumping; cessation of shape changes
4	9.00	63	"Normal" mitochondria
1	7.45 (end)	70	"Normal" mitochondria

of mitochondria changed rapidly in acid conditions. Table II illustrates the effect of varying the pH of the medium. Hydrogen ion concentration was ascertained immediately after each homogenization, and in all cases had been maintained between pH 7.4–7.6.

The method of homogenization proved to be an important factor in preserving the structure of the mitochondria. Table III illustrates the differences in numbers and kinds of particles observed after rupturing the cells by different methods. The syringe method is probably not optimal, however, since a certain amount of foaming is unavoidable. Undoubtedly, failure to replicate homogenization procedures from one sample to another is one of the greatest sources of variation in the data obtained from different homogenates.

TABLE III

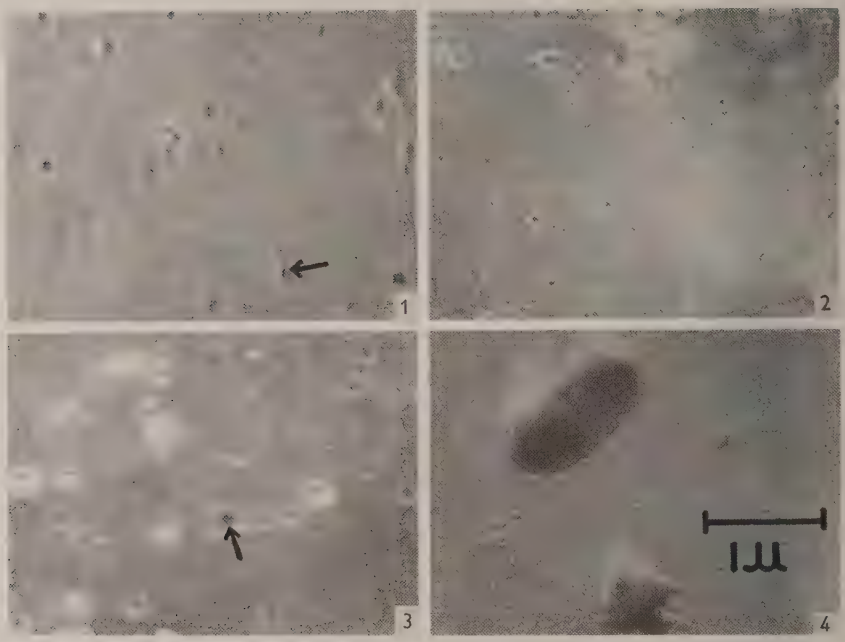
Effect of method of homogenization on mitochondrial structure.
S. purpuratus, unfertilized eggs, homogenized in buffered 1.1 *M* sucrose.

Method	No. of particles per 0.001 mm^3 (Av. of 5 chambers)	Size and appearance of particles
Blendor	33	0.2 μ; rods and small granules
Glass homogenizer (with ground glass pestle)	20	0.2–0.5 μ; refractile bodies and "ghosts"
Syringe	17.5	0.3–2.0 μ; bipartite spheres and ovals

The degree of accuracy of the method also depends on the ability of the observer to identify mitochondria in the homogenates. Preliminary studies suggested that six criteria could be consistently applied in the identification of a cytoplasmic inclusion as a mitochondrion.

Size.—Spherical or oval forms between 0.5 and 2.5 μ in diameter were counted, the majority measuring 1.5 μ; rod forms varied in size between 0.75 and 2.0 μ in length, the greater number being slightly longer than 1.0 μ. The adoption of these size ranges eliminated smaller particles, some of which may have been mitochondria, but the difficulty in applying the other criteria for mitochondria to very small particles made their positive identification too uncertain.

Structure.—Many of the spherical or oval forms observed had the bipartite structure which has been described for mitochondria of mammalian liver



Figs. 1 3. Photographed with phase contrast microscope (dark *M* oil immersion objective), × 1030.

Fig. 1. *L. pictus*. Mitochondrial fraction of unfertilized egg homogenate, sedimented at 14,140 *g* for 20 min.; resuspended in 1.1 *M* sucrose, pH 7.5. Note variations in size of mitochondria; bipartite structure (see text) can be seen in one mitochondrion (arrow).

Fig. 2. *L. pictus*. Fraction sedimented from prism larvae at 1120 *g* for 10 min. resuspended in 1.1 *M* sucrose, pH 7.5. Photograph somewhat less enlarged than Fig. 1. Short rod forms are more abundant at this stage.

Fig. 3. *S. purpuratus*. Homogenate of unfertilized eggs, diluted 1:9 with 0.25 *M* sucrose, pH 7.6. Mitochondrion indicated by arrow has swollen three or four times normal volume; note denser peripheral portion.

Fig. 4. Electron micrograph of mitochondrion in homogenate of *L. pictus* gastrula. Homogenate dialyzed on collodion membrane, fixed in 2 per cent osmium tetroxide (pH 7.4), air dried, chrome shadowed. Original magnification × 11,200.

cells [8, 16]. This consists of a sphere or oval, at the periphery of which is a denser body (Fig. 1). These forms were most numerous in eggs and early embryos. Dumb-bell shaped forms, rods with knobbed ends, and cylindrical rods were also observed, the latter especially in later stages (Figs. 2 and 4).

The intensity of phase contrast of mitochondria was unlike that of other types of cytoplasmic inclusions seen in the homogenates (pigment granules, yolk spheres, oil droplets, and very small fibrillar elements). Spherical or

oval mitochondria appeared as medium to dark grey in hue, with the peripheral portion much darker; rod forms were uniformly a deep gray to black.

The behavior of mitochondria in media of different osmotic concentrations typically distinguished them from other inclusions. Preliminary observations showed that spherical or oval forms swell as the medium becomes more hypotonic, and eventually burst, leaving a 'ghost' to which adheres the darker peripheral body (Fig. 3). Rods also swell in these conditions, and may break transversely.

Living mitochondria undergo *shape changes* which other types of inclusions do not exhibit. Under acid conditions, the cessation of these changes is very striking.

An aid in distinguishing particles other than mitochondria in the homogenates was their *relative numbers* in different stages of the same species, or in the same stage of the two species. For example, *Lytechinus* eggs and embryos have fewer pigment granules than do those of *Strongylocentrotus*. Also, yolk spheres decrease in number in later stages of both species.

Regardless of the rigorousness with which the criteria for identification of mitochondria are applied, however, it is apparent that subjective error may influence the counts. A preliminary series of counts was made by two observers on samples from homogenates of different stages, in different dilutions. Table IV illustrates the results of two observers counting mitochondria in the same chamber for each of two dilutions. The figures are typical of the degree of difference in the counts made by the two observers, but there did not seem to be any consistent tendency for one observer to register higher or lower counts than the other. All of the results presented represent counts by one observer.

TABLE IV

Mitochondrial counts of two observers on same sample.
S. purpuratus, 123 hrs. (17°C), homogenized in buffered 1.1 *M* sucrose.

Dilution	Observer	Counts per 4 small squares (Av. of two counts)	Total
Undiluted	*a*	18.5 22.0 20.0 18.5 16.0	95.0
	b	22.5 24.5 29.5 35.0 24.5	136.0
1:1*	*a*	11.5 14.5 14.0 15.5 12.0	67.5
	b	14.5 10.0 10.0 12.5 8.5	55.5

* Diluted with 1.1 *M* sucrose.

The data obtained from the counts of embryos and mitochondria were analyzed statistically, as follows:

Beginning with a suspension containing a certain number of eggs or embryos, homogenates of which contain a certain number of mitochondria, one may wish to be able to withdraw samples from these suspensions and compute from these samples two numbers, such that the probability that the number of mitochondria per egg or embryo lies between these two numbers is at least some preassigned amount. One method of doing this is given below.

Suppose that the original suspension is of volume V, and that there are N_1 embryos and N_2 mitochondria in it. The suspension is diluted by a factor c, and a number of independent samples of volume v are withdrawn. The number of embryos in each sample is counted. It is known that the probability that exactly k embryos will be found in a sample is

$$\frac{\left(N_1\frac{v_1}{c_1 V}\right)^k e - \left(N_1\frac{v_1}{c_1 V}\right)}{k!}$$

(Poisson distribution).

The suspension of embryos is homogenized and diluted by a factor c_2. Samples of volume v_2 are counted for the number of mitochondria. The probability that exactly j mitochondria are found in the samples is given by

$$\frac{\left(N_2\frac{v_2}{c_2 V}\right)^j e - \left(N_2\frac{v_2}{c_2 V}\right)}{j!}$$

The expected number of mitochondria in a sample of volume v_2 from the diluted suspension is $N_2(v_2/c_2 V)$, and the expected number of embryos in a sample of volume v_1, from the first diluted suspension, is $N_1(v_1/c_1 V)$. Thus, the ratio of the expected values is $c_1 v_2/c_2 v_1$ times the number of mitochondria per embryo.

A procedure for obtaining confidence intervals for the ratio of the expected values from two independent Poisson distributions has been given by Chapman [2]. In this particular case, the procedure is to take n samples from a suspension of embryos and let X equal the total number of embryos counted. The embryos are then concentrated, resuspended and homogenized, and n more samples are counted for mitochondria, and the number in the total suspension, Y, obtained. Chapman [2] points out that the probability distribution of X, given X plus Y, is a binomial distribution with parameter $N_1 c_2 v_1/(N_1 c_2 v_1 + N_2 c_1 v_2)$. Confidence intervals for this parameter are well known, and can be found in Eisenhart, Hastay and Wallis [3]. If (a_1, a_2) forms a confidence interval for $N_1 c_2 v_1/(N_1 c_2 v_1 + N_2 c_1 v_2)$, then $c_2 v_1/c_1 v_2 a_2 - c_2 v_1/c_1 v_2$, $c_2 v_1/c_1 v_2 a_1 - c_2 v_1/c_1 v_2)$ forms a confidence interval for N_2/N_1, the number of mitochondria per embryo.

An illustrative example may be quoted here. From initial suspensions of 40 ccm³ of embryos, 1 ccm³ was withdrawn and diluted to 250 ccm³ with sea water; five 0.5 ccm³ samples were withdrawn and the number of embryos counted. The same

procedure was followed for a second sample withdrawn from the same suspension. Data are given below for two suspensions of *S. purpuratus*, 30 hours old at 17°C.

Suspension	*a*		*b*	
Sample	1	2	1	2
	18	14	19	24
	16	22	20	17
	18	26	17	37
	20	20	20	27
	17	15	20	21

The total numbers of embryos counted in the samples from each suspension were 186 (*a*) and 222 (*b*). The suspensions were concentrated, resuspended in 40 ccm³ of buffered sucrose; (1.1 *M*), and the embryos homogenized; 0.5 ccm³ was withdrawn from the homogenate and diluted to 5 ccm³ with the sucrose medium; samples of this dilution were placed in the counting chamber, and the number of mitochondria in 0.001 ccm³ (20 smallest squares of the ruled grid) were counted in each corner and in the center of the central square of the grid. The mitochondrial counts obtained from the homogenates of the embryo suspensions noted above were:

Homogenate	*a*		*b*	
Sample	1	2	1	2
	19	18	21	27
	14	17	18	22
	19	15	21	13
	12	14	16	20
	17	12	12	17

The total numbers of mitochondria counted in the samples from each homogenate were 157 (*a*) and 187 (*b*). The total number of embryos plus the total number of mitochondria counted were 343 (*a*) and 409 (*b*). The ratios of mitochondria to the total numbers of embryos and mitochondria were then computed, giving $\frac{186}{343} = 0.45$ (*a*) and $\frac{222}{409} = 0.54$ (*b*). Looking at the tables for 90 per cent confidence limits for binomial random variables with sample per cent 0.54 and samples sizes 343 and 409, the limits $a_1 = 0.46$ and $a_2 = 0.62$ (*a*) and $a_1 = 0.47$ and $a_2 = 0.61$ (*b*), were obtained.

Computing $c_2v_1/c_1v_2a_2 - c_2v_1/c_1v_2$ and $c_2v_1/c_1v_2a_1 - c_2v_1/c_1v_2$ for $c_1 = 250$, $c_2 = 10$, $v_1 = 0.5$ and $v_2 = 10^{-6}$, the limits $14.4 - 21.6$ (*a*) and $15.1 - 20.0$ (*b*) were established.

Figs. 5 and 6 represent individual homogenates of embryos of different ages, of the two species studied. Each point on the graphs is the mid-point of the confidence interval, calculated as explained above. Figs. 7 and 8 represent the confidence limits (at 90 per cent level) calculated from the combined data from all homogenates of embryos of the same age.

These results may be briefly summarized: (1) Fluctuations in mitochondrial populations occurred in the two species studied at the same stages (allowing

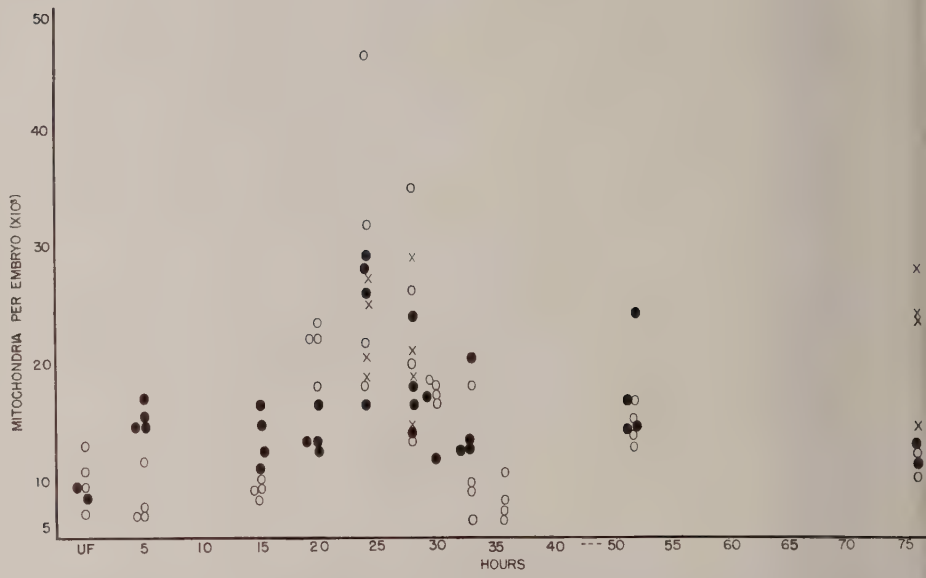


Fig. 5. *S. purpuratus* (17°C). Determinations of mitochondria per embyro from individual homogenates (see text). The different symbols at each age represent counts on sets of homogenates prepared in the same experiment.

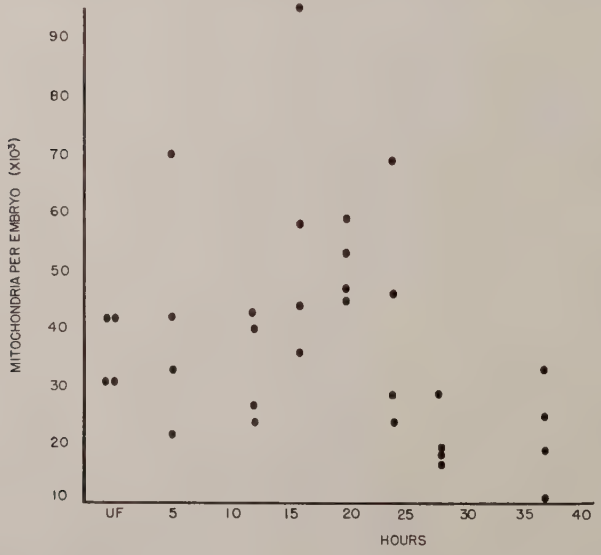


Fig. 6. *L. pictus* (19.5°C, av.). Determinations of mitochondria per embryo from individual homogenates (see text).

for difference in temperature of culture). (2) There did not appear to be any significant change in the numbers of mitochondria per embryos during the cleavage and blastula periods (up to ca. 15 hrs. for *S. purpuratus*, and to ca. 12 hrs. for *L. pictus*). (3) At the time when primary mesenchyme cells first become apparent in the blastocoele (ca. 20 hrs. for *S. purpuratus*, 16 hrs. for *L. pictus*), an increase in the number of mitochondria commenced, reaching a peak in the early gastrula (24–28 hrs. for *S. purpuratus*, 20 hrs. for *L. pictus*). (4) This increase in numbers of mitochondria per embryo was followed shortly by a sharp decrease in mitochondria, extending through the completion of gastrulation and the formation of the prism larva (30–36 hrs. for *S. purpuratus*, 24–37 hrs. for *L. pictus*). (5) In *S. purpuratus* later stages (52 and 76 hrs.; pluteus larvae) had mitochondrial populations about equal to those found in the unfertilized egg.

DISCUSSION

The volume of the unfertilized egg occupied by particles identified as mitochondria may be calculated, assuming the following values: average diameters of eggs of *S. purpuratus* and *L. pictus* as 111 and 80 μ respectively; volume of a mitochondrion based on a sphere of average diameter of 1.5 μ; average number of mitochondria per egg taken as the midpoints of confidence intervals (Figs. 7 and 8; 14,000 and 33,000 for *S. purpuratus* and *L. pictus* respectively). Values of 9.2 and 8.4 per cent for *S. purpuratus* and *L. pictus* respectively, are obtained. These are about twice the value reported by E. N.

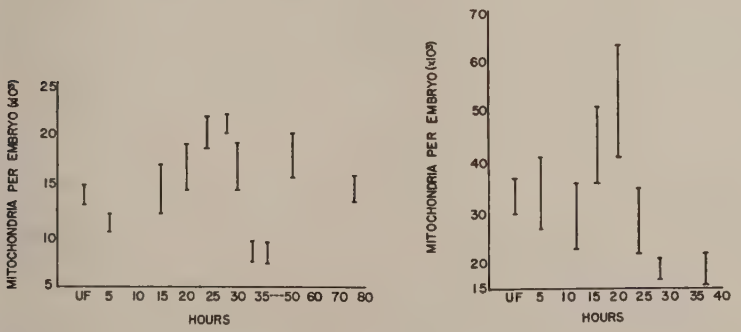


Fig. 7. *S. purpuratus* (17°C). Confidence limits for number of mitochondria per embryo; combined data from all homogenates of one age (see text).
Fig. 8. *L. pictus* (19.5°C, av.). Confidence limits for number of mitochondria per embryo; combined data for all homogenates of one age (see text).

Harvey [9] for *Arbacia punctulata* (4.8 per cent), which was calculated on the basis of the volume of a spherical section occupied by mitochondria in a centrifuged egg.

Few estimates of the numbers of mitochondria in individual animal cells are available. Allard, Mathieu, de Lamirande and Cantero [1] report a number of 2500 mitochondria per normal rat liver cell. Larger numbers of mitochondria might be expected to occur in a relatively large cell such as the sea urchin egg, but it is apparent that, assuming equal distribution of particles to all cells during cleavage, the number of mitochondria per cell becomes increasingly smaller. However, in the stages where a sharp decrease in the number of mitochondria per embryo was noted (gastrulae and prism larvae), observations on intact embryos has indicated [14] an increase in the numbers of mitochondria in cells of the forming gut and in ciliated cells. There appeared to be a concurrent diminution in the number of mitochondria in the cytoplasm of body wall cells. Thus, while the total number of mitochondria per embryo may become very small in these stages, the numbers in particular kinds of cells may actually rise. It has been suggested [14, 15] that such localizations of mitochondria might reflect the physiological activity of these cells of the embryo at this time.

Recent studies [12, 13] indicate that the chemical composition of mitochondria may vary with their size. Although no differential counts were made in the present investigation to ascertain the numbers of mitochondria of different sizes within the limits adopted, the impression was gained that the larger spherical or oval forms, representing the majority of mitochondria in eggs and early stages, were superseded by small rod forms in later stages. It is possible that transitions in size classes of mitochondria occur during development of the sea urchin. However, Shaver [15] was unable to see any evidence of the formation of mitochondria by the aggregation of smaller elements, as has been reported in another sea urchin species [6, 7].

SUMMARY

A method for the estimation of mitochondrial numbers during sea urchin development, based on counts of these structures in homogenates of successive stages of two species, is described. Some details of the optimal conditions for homogenization of the material, and of the criteria for identification of mitochondria by phase contrast microscopy, are given. A method for the statistical analysis of the data is presented. The numbers of mitochondria remain approximately constant during cleavage and blastula stages. When primary

mesenchyme cells begin to migrate into the blastocoele, an increase in the number of mitochondria was observed, reaching a peak in the early gastrula. In immediately succeeding stages, the number of mitochondria dropped sharply. In one species, a rise in mitochondrial numbers was noted in pluteus stages. Some conclusions regarding the numbers of mitochondria per cell in developing embryos of the sea urchin are discussed.

The author wishes to express his appreciation to Prof. Albert Tyler for reading the manuscript; to Miss Mary Jones for technical assistance; and especially to Dr. D. R. Truax, Mathematics Department, California Institute of Technology, for the statistical treatment of the data.

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THE EFFECT UPON MITOSIS IN *ALLIUM CEPA* OF AN ANTIFUNGAL ANTIBIOTIC PRODUCED BY *STREPTOMYCES* SP.

R. CRAVERI and U. VERONESI

Montecatini Antibiotic Research Laboratories and National Cancer Institute, Milan, Italy

Received May 22, 1956

In the course of researches upon the inhibition of tumours by antibiotics, many of these substances have been discovered to be mitotic poisons [7, 8, 9, 11, 12, 14, 15, 16, 17]. The best clinical results appear to have been achieved by Actinomycin *C* [5, 13].

The antimitotic effects of a series of antibiotics have been tested in our laboratories, and some of these have already been described [10]. The present study is concerned with the effect upon mitosis in root meristems of *Allium cepa* of an antibiotic (antibiotic 757) produced by a species of *Streptomyces*.

METHODS AND MATERIALS

The methods of production and isolation of antibiotic 757, which closely resembles Ascosin, described by Hickey *et al.* [6], will be the subject of a separate communication, and are given here only in outline.

Streptomyces strain No. 757 was grown for six days at 26–28°C in a medium containing mannitol, peptone, starch and corn-steep liquor, in 300 ml flasks, agitated in a shaker. The final pH was approximately 8.5.

The broth filtrate was extracted with butanol, from which the antibiotic was obtained by concentration *in vacuo*, removal of the precipitate from the solute by centrifugation, and repeated washing in ether. The yield was 400 mg/l approx.

The product is a light yellow, amorphous powder, insoluble in water, slightly soluble in ethanol and methanol, and very soluble in pyridine and alkalis. Aqueous solutions may thus be prepared by dissolving in dilute NaOH and further diluting with distilled water. The calcium and magnesium salts of tapwater caused rapid precipitation.

Antibiotic 757 remains stable for several months if stored *in vacuo*, in the dark. The aqueous solution is unstable to light, especially ultraviolet, but remains active if kept in the dark, at room temperature, at pH 6–10, for about ten days. The ultraviolet absorption spectrum of an ethanol solution at 40 mcg/ml showed maxima at 285, 361, 381 and 404 mmc.

Antibiotic 757 is extremely active against yeasts and filamentous fungi at concentrations of 0.01–0.1 mcg/ml. It is inactive against bacteria. LD_{50} to mice is ap-

proximately 5 mg/kg intraperitoneally. Much larger doses can be withstood subcutaneously, and these cause extensive local necrosis, as in the case of Sarkomycin [15].

Preliminary tests upon wheat and oats having indicated that very low concentrations of the antibiotic retarded the growth of the roots and interfered with mitosis, a more extensive series of tests was undertaken upon *Allium cepa*, in which crushed preparations of root tips provide excellent cytological pictures.

Experiments were conducted during the winter months. Bulbs 5 cm in horizontal diameter were permitted to germinate upon 100 ml beakers (4 cm in diameter) containing distilled water, at 20°C. The beakers were screened with black paper.

After the three days necessary for root development had elapsed, varying quantities of a 5 per cent w/v solution of the antibiotic in *N*/10 NaOH were added to the water. In the case of controls, an equivalent quantity of NaOH alone was added. Since the growth of the onion slightly acidified the water, the resulting solutions were approximately neutral.

The concentrations of antibiotic 757 tested were 5, 10, 20, 50, 100, 200, 500 parts per million, for periods of from 15 to 96 hours. After these periods the onions were transferred to the water from which the corresponding controls had been removed, and the process of recovery was examined after intervals of 6, 24 and 48 hours. Some precipitation of the antibiotic occurred after 24 hours at solutions of 50 p.p.m. and over.

The root growth, mitotic index and mitotic formula were evaluated, and other cytological changes in the root meristem cells observed. For staining purposes acetocarmine and Feulgen's method were used, especially the latter.

OBSERVATIONS

Growth.—Concentrations of 5 parts per million produced marked retardation of root development, by comparison with the controls. As the concentra-

Table I

Root lengthening of *Allium cepa* bulbs treated with antibiotic 757

(Averages from three bulbs; in cm.)

Concentration	Time of treatment, hrs.			
	24	48	72	96
Untreated	0.7	1.6	2.5	3.2
5 p.p.m.	0.4	0.6	0.9	1.5
10 p.p.m.	0.4	0.4	0.7	1.2
20 p.p.m.	0.3	0.3	0.5	0.7
50 p.p.m.	0.3	0.3	0.5	0.6
100 p.p.m.	0.3	0.3	0.4	0.6
200 p.p.m.	0.3	0.3	0.3	0.4
500 p.p.m.	0.2	0.2	0.3	0.4

TABLE II

Mitotic index ‰ in the cells of *Allium cepa* root tips treated with antibiotic 757.

Concentration	Time of treatment, hrs.				
	15	24	48	72	96
Untreated	113	115	144	142	136
5 p.p.m.	68	61	42	31	48
10 p.p.m.	64	55	26	23	42
20 p.p.m.	47	31	17	8	16
50 p.p.m.	41	12	7	11	12
100 p.p.m.	29	10	6	10	9
200 p.p.m.	20	8	5	3	2
500 p.p.m.	16	10	4	2	2

TABLE III

Mitotic formula (%) in the cells of *Allium cepa* root tips treated with antibiotic 757.

Concentration	Time of treatment, hrs.																			
	15				24				48				72				96			
	P	M	A	T	P	M	A	T	P	M	A	T	P	M	A	T	P	M	A	T
Untreated	52	22	14	12	56	23	12	9	49	26	14	11	48	25	15	12	47	27	16	10
5 p.p.m.	45	31	15	9	40	42	10	8	31	50	11	8	43	37	11	9	46	30	13	11
10 p.p.m.	41	39	12	8	34	54	7	5	16	63	13	8	39	42	11	8	37	35	15	13
20 p.p.m.	27	59	9	5	17	76	4	3	7	87	3	3	20	68	7	5	30	42	16	12
50 p.p.m.	27	46	17	10	28	47	14	11	14	71	7	8	28	51	12	9	30	40	17	13
100 p.p.m.	25	49	16	10	22	58	11	9	16	70	8	6	24	62	9	5	27	49	16	8
200 p.p.m.	22	54	14	10	20	61	10	9	13	75	7	5	21	65	11	3	25	63	8	4
500 p.p.m.	20	61	10	9	15	69	9	7	10	85	3	2	8	87	3	2	11	82	5	2

P = Prophases; M = Metaphases; A = Anaphases; T = Telophases.

tion was increased, this effect was correspondingly intensified, until at 200 to 500 parts per million many of the root tips became soft, flaccid and fragile, and remained so far as long as the treatment was continued (Table I). Small swellings, reminiscent of those produced by colchicine, appeared at the tips of many roots, and after about a week's exposure to the antibiotic similar swellings developed at intervals along the roots; after a further period of two or three days, these developed into branch-roots (Fig. 2).

Mitosis.—In Tables II and III and Fig. 1 are shown the mitotic index, mitotic formula and metaphase percentage for the roots of bulbs subjected to the action of the antibiotic. Each datum refers to the mean of six root tips, two from each of three bulbs. In the case of concentrations of 200 and 500 parts per million, observations were made upon those tips which had not

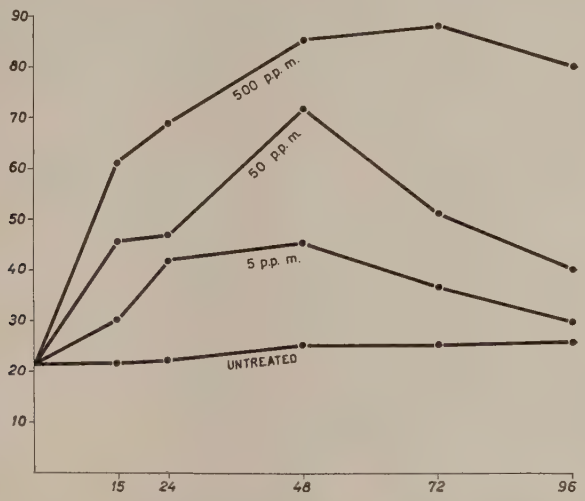


Fig. 1. Graph showing the percentage of metaphases in root meristem of *Allium cepa* after treatment with three concentrations of antibiotic 757.

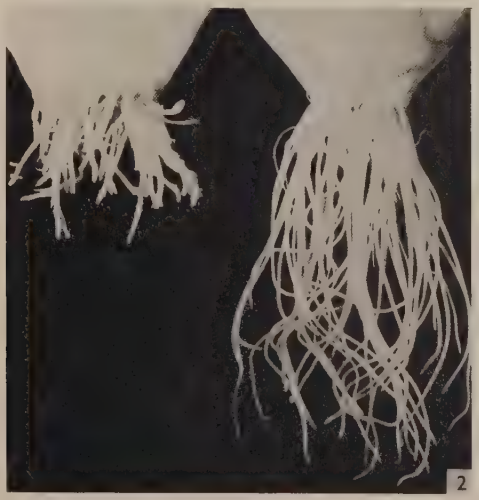
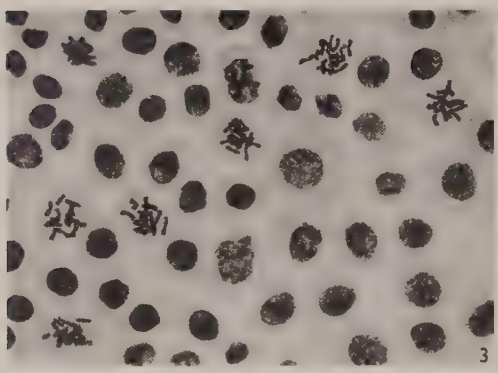
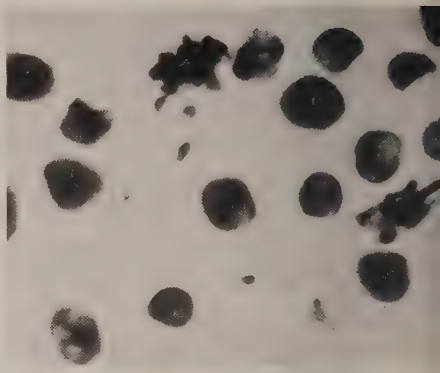


Fig. 2. *Left*, onion roots 7 days after the beginning of treatment with antibiotic 757. (20 p.p.m.). *Right*, untreated.





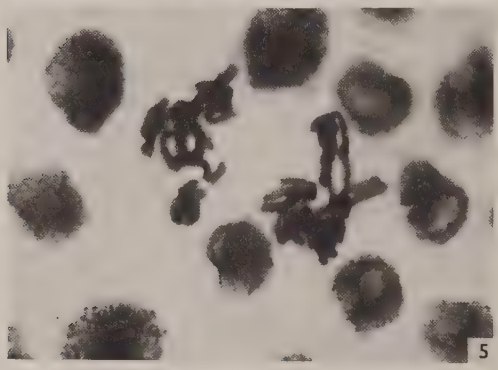


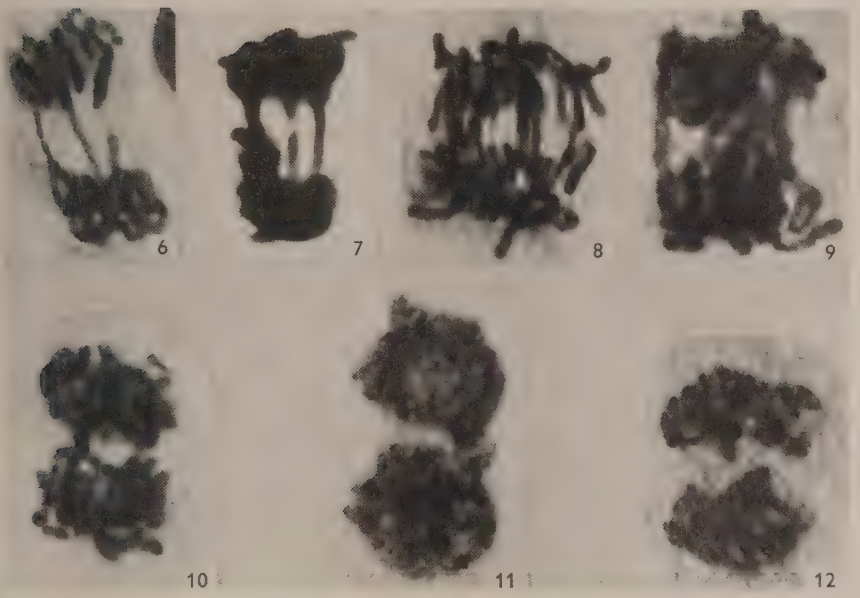
Fig. 3. Several metaphases showing C-mitosis feature (after 48 hrs. treatment with 20 p.p.m. conc.).
Fig. 4. Severe damage with nuclear fragmentation (after 72 hrs. treatment with 100 p.p.m. conc.).
Fig. 5. Irregular prometaphases (after 24 hrs. treatment with 10 p.p.m. conc.).

yet fallen away, i.e. at 72 hours, about a third of the original total. Polyploidy of the nucleus was not observed.

On transfer to the antibiotic-free water from which the controls had been removed, no recovery on the part of the test roots could be detected after six hours. After 24 hours the mitotic index began to revert to normal; and after 48 hours mitotic activity was apparently completely restored. However, the radiomimetic changes which will be described later were still very manifest at this stage.

The mitotic disturbances caused by antibiotic 757 were of five main types:

(1) *Primary cytotoxic effect*, taking the form most usually of chromosome stickiness, which led to the formation of chromosome bridges at anaphase and telophase, and occasionally of pseudochiasmata (Figs. 6–12). These appearances were first observed after as little as two hours exposure to the



Figs. 6–12. *6*, *7*, *8*, *9*, Different anaphases with sticky bridges. *10*, Telophase with chomatinic isthmus preventing separation of the two daughter nuclei. *11*, Resting cell with dumbell-shaped nucleus. *12*, Binucleated cell immediately after nuclear division.

lower concentrations of the antibiotic, but were delayed as much as eight hours in the case of the higher concentrations, presumably because the onset of mitosis was also delayed.

(2) *C-mitosis.*—This phenomenon was observed with all concentrations of antibiotic, the onset being between the fifth and tenth hour of treatment. The appearance was typical, except at the highest concentrations, where sticky metaphases occurred. The more or less complete inactivation of the spindle, responsible for *C*-mitosis, led to an increase in the proportion of metaphases (Fig. 1). With low concentrations, some of the *C*-mitoses continued the normal mitotic course while others returned directly to the resting stage. At high concentrations, *C*-mitoses became pycnotic and eventually died.

(3) *Radiomimetic* (*nucleotoxic*) *effect.*—This was shown by the presence of both anaphasic chromosome bridges and acentric fragments, and was observed mainly at high concentrations of antibiotic, commencing at about the fifteenth hour. The proportion of cells involved was very small. Sometimes fragmentation led to the occurrence of micronuclei in resting cells (Fig. 4).

(4) *Secondary cytotoxic effect.*—Severe lesions, with advanced chromosome fragmentation, nuclear degeneration and spindle abnormalities were produced by the highest concentrations. These were suggestive of an effect upon both chromosomes and cytoplasm.

(5) *Statmodiaeresis.*—This effect, usually produced by purine derivatives, entails the inhibition of cell wall formation between daughter nuclei at telophase, and results in the production of binucleate cells. A large number of such cells appeared, especially at 24–48 hours after commencement of treatment. It is our opinion that these did not result from the dissolution of the bridges in the incompletely divided nuclei described in section 1.

CONCLUSIONS

The decrease in the mitotic index in root cells continuously treated with antibiotic 757 is indicative of an inhibitory action upon the onset of prophase. This effect is apparent at very low concentrations and may represent an interference with the metabolism of the preprophase [1, 2, 3, 4]. Some action upon the interphase nucleus is also indicated by the constant, if moderate, radiomimetic effect. The inhibition of the spindle led to an increase in metaphase rate, including some *C*-mitosis. In connection with this phenomenon, although some authors confine this term to those *C*-mitotic appearances which result in polyploidy, the very characteristic appearance of these figures, as already remarked, justifies our use of the name.

The most interesting features of the action of this antibiotic are its remarkable antimitotic activity at very low concentrations, and its apparently specific effects upon different parts of the cell. By comparison with 'Actinomycin' previously studied [5, 10, 13], the growth inhibiting activity upon *Allium* roots is almost the same, whereas antibiotic 757 is about twenty times less toxic to mice.

SUMMARY

The effects upon *Allium cepa* roots of an antibiotic (antibiotic 757), produced by a species of *Streptomyces*, are described. Root growth is inhibited at low concentrations, the mitotic index is lowered and a variety of pathological conditions develops in the mitotic nucleus. The metaphase percentage is greatly increased, *C*-mitosis is induced, and at higher concentrations radiomimetic and statmodiaeretic effects are observed.

The authors wish to thank Professors K. A. Bisset, G. Giolitti and C. Sirtori for their advice and interest.

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QUANTITATIVE DETERMINATION OF CHOLINESTERASE ACTIVITY IN INDIVIDUAL CELLS

J. ZAJICEK and E. ZEUTHEN

Institute of Radiopathology, Radiumhemmet, Stockholm, Sweden, and Zoophysiological Laboratory, University of Copenhagen, Denmark

Received June 5, 1956

In the course of studies [12, 13] on the cholinesterase activity (ChE) of blood cells (platelets and megakaryocytic structures) the need arose for a micromethod for quantitative determination of the ChE activity of single megakaryocytes.

Considerations too involved for present discussion led us to assume that the acetic acid formed in the enzymatic hydrolysis of acetylcholine (ACh) by a single mature megakaryocyte liberates from the bicarbonate buffer about 5×10^{-4} μl CO_2 per hour. The enzyme activity of the megakaryoblast was expected to be about one tenth of this value.

The most sensitive method hitherto evolved for ChE determination is the Cartesian diver microgasometer of Linderstrøm-Lang [6]. With this apparatus analyses were made of serum samples [9] and tissue fragments [1] with ChE activity corresponding to the liberation of about 0.01 to 0.1 μl CO_2 per hour.

The following paper describes a Cartesian diver method which permits quantitative determination of enzyme activity in single megakaryocytes of rat bone marrow. The method is a modification of the ampulla-diver respirometer used for measuring the respiration of single protozoan cells [16].

MATERIAL AND METHODS

Substrate. –The substrate found to meet the requirements for both stability and specificity was the thioanalogue of ACh, acetylthiocholine (AThCh). This ester was first synthetized by Renshaw *et al.* [10], who noted that its pharmacologic actions were similar to those of ACh, but were of briefer duration. Glick [2] reported that at pH 8 AThCh underwent more rapid hydrolysis, both enzymatically and nonenzymatically, than ACh. This finding was confirmed by Koelle and Friedenwald, who also introduced the thiocholine esters as substrates for the histochemical demonstration of ChE and acetylcholinesterase (AChE) [3, 4]. With a modified thiocholine method it was demonstrated histochemically that the esterase found in the megakaryocytes was AChE [14]. This enzyme displays optimum activity at AThCh concentrations ranging from 4×10^{-3} to 6×10^{-3} M [3]. We therefore decided to

measure the esterase activity in megakaryocytes with 6×10^{-3} *M* AThCh as the substrate (Acetylthiocholine iodide, F. Hoffmann-La Roche, Basle).

The composition of the incubation solution used is given in Table I.

TABLE I

	Solution % (w/v)	ml
NaCl	0.8	10
$NaHCO_3$	0.28	10
$CaCl_2 \cdot 6H_2O$	1.34	0.4
	Total	20.4

The quantity of AThCh dissolved to make the concentration 6×10^{-3} *M* was 35.4 mg. The pH of the AThCh-bicarbonate buffer solution when saturated with a mixture of 5 per cent CO_2 and 95 per cent N_2 is about 7.4 [11].

Preparation of bone-marrow cell suspension. Bone-marrow cells from the femur of an 8-week-old albino rat were suspended in a solution of 5 per cent sodium citrate

Fig. 1. Polymorphonuclear megakaryocyte isolated from rat bone marrow and placed in diver.

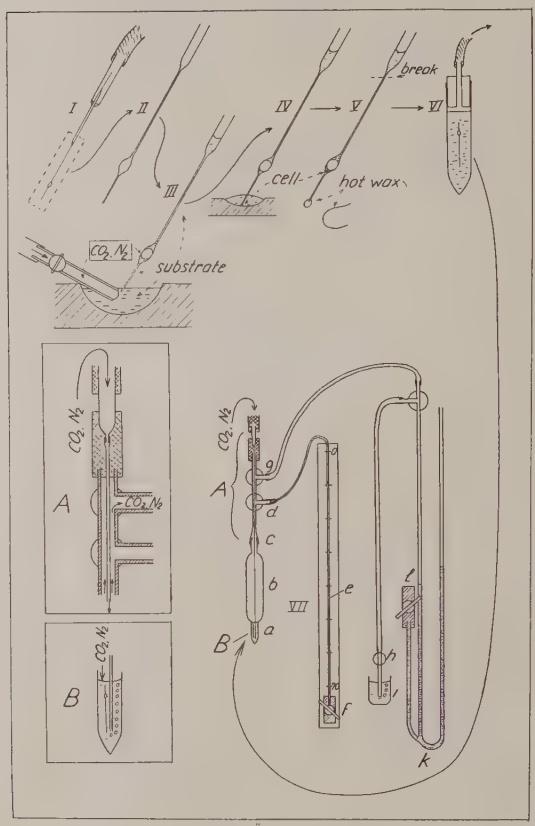

Fig. 2. *I.* The braking pipette: A capillary (diameter 0.5 mm) is pulled from a thin-walled test tube and is fitted into a glass jacket, using De-Khotinsky cement. The pressure is regulated by mouth, through rubber tubing. Details inside the dotted frame are enlarged in *II* to *V*.

II. The lower portion of the braking pipette forms an "ampulla" approximately 3 mm long with a "shaft" measuring about 20 mm and a "tip" of about 10 mm. The internal diameter of the shaft and the tip is 40 to 60 μ.

III. Substrate previously bubbled through with 5 per cent CO_2 and 95 per cent N_2 is sucked into the ampulla, shaft and adjacent parts of the pipette. This is followed by a bubble of the same gas mixture, taken from the slanted tube to the left (during interruption of the flow). Finally substrate is sucked into the tip and the lower part of the ampulla, leaving gas and fluids inside the diver in the positions indicated.

IV. The cell is deposited in a droplet on an agar-coated hollow slide.

V. The cell is sucked up to the position shown. The tip is sealed with a resin–wax mixture (1 part resin, 2 parts wax) heated to about 80°C. Breaking of the shaft at the site indicated separates the charged ampulla-diver from the pipette. The latter now has a sealed tip and what was the shaft becomes the "tail". The diver is transferred to a special vessel (*VI*) containing CO_2-N_2-bubbled substrate.

VI. If the diver floats to the surface the stopper is inserted and the gas space may be reduced stepwise until buoyancy is attained at, or in the vicinity of, atmospheric pressure. This can be accomplished by suction (arrow), with return to atmospheric pressure whenever a small bubble is observed to escape through the tail of the diver. By repeated suction, however, the liquid in the diver is depleted of gases and the time required for equilibration may thus be prolonged. We therefore preferred to work with slightly overweight divers, i.e. divers with excessively long tails (2 cm) and to regulate buoyancy by progressively shortening the tails. From *VI* the diver is transferred to the flotation vessel of the "manometer" (*VII*), disconnecting and reassembling being carried out at *c*.

The manometer is really a burette (*d*, *e*, *f*), by means of which accurately measured volumes of fluid can be withdrawn from, or injected into, a closed air space (*b*), thereby creating regulated pressure changes in the space. In our apparatus the approximate calibration of the burette is 1 μl/mm and that of the air space *b* with all ramifications 200,000 μl. A 1-mm shift of the meniscus *e* in the burette therefore corresponds to a pressure change of 5×10^{-6} atm. or 0.05 mm H_2O. The diver floats in substrate in a pocket *a* which opens to *b*. The assembled manometer is fixed at the edge of a special ($\pm$ 0.002°) water bath (supplied by O. Dich, Avedøre, Copenhagen), in

(1 part) and 0.8 per cent sodium chloride (4 parts). As the anticoagulant citrate is an inhibitor of ChE, the cells were washed free of citrate by centrifugation and were placed in 0.9 per cent sodium chloride. Centrifugation at 1500 rpm for about 45 seconds then produced a fraction rich in megakaryocytes. A small drop of this fraction was resuspended in 0.5 ml of AThCh-bicarbonate buffer solution.

Isolation of individual megakaryocytes. –A few drops of the bone-marrow cell suspension in AThCh-bicarbonate buffer were placed on a hollow slide. Megakaryocytes, like platelets, display a strong tendency to adhere to glass. This makes the handling of individual megakaryocytes very difficult, if not impossible. To overcome this difficulty we first tried, without success, coating the slides with paraffin or silicone. It was finally found that agar gave the desired effect. On an agar-coated hollow slide the megakaryocytes behave as though freely suspended. The cell to be investigated can thus readily be sucked into a braking pipette and transferred to another agar-coated slide. When the accompanying erythropoietic or granulopoietic cells have been pipetted off, the megakaryocyte is sucked into the diver (Fig. 1), to the position indicated in Fig. 2, V. With a little practice all these operations can be performed under a standard light microscope, without the aid of a micromanipulator.

The manometric measurement of CO_2.—The method for determining ChE is best described graphically. Fig. 2 and its extended legend give an impression of how the method functions. The reader is therefore advised at this point to consult Fig. 2.

The method discussed is for a standard diver, gas volume 0.5 μl, liquid charge 0.6 μl. It is easy to charge divers with less than 0.6 μl of liquid, and indeed in most of our experiments the charge was only about 0.2 μl. A large liquid charge, however, causes increase of the initial period (t_s, cf. p. 575) before gas formation or gas uptake can be registered manometrically. The standard diver then provides less favourable experimental conditions. Since the absorption coefficient for CO_2 in water at 22°C is 0.83, our standard diver compares with a 1 μl-diver without liquid charge.

The diver floats only if made of glass so thin that it readily cracks during manipulation with fingers. In all the steps of making and filling, however, the diver remains attached to the braking pipette capillary from which it is pulled, and need not be touched by hand. Our divers were made from thin-walled test tubes (Jena glass) pulled in several operations to the dimensions indicated in Fig. 2. It is not essential that the cross section of the capillary (diameter ca. 0.5 mm) used for the braking pipette be circular. Most often, in fact, it is not. The "shaft" and "tip" of the "ampulla" (cf. legend, Fig. 2) are pulled in a microflame without rotation of the glass. The shaft becomes the "tail" of the diver. The glass is heated from one side when the

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which *a*, *b*, *c* and *d* are submerged. The manometer is perfused with 5 per cent CO_2 and 95 per cent N_2 through narrow polyethylene tubing, which is introduced deep into the pocket *a* (cf. insert *B*). The tubing passes the 3-way stopcocks *g* and *d* above the flotation vessel (insert *A*). The flow is continued for 20 minutes at an approximate rate of 50 ml/min. The gas escapes at *h*, *i*. Meanwhile the burette (*d*, *e*) and the water manometer (*k*, *l*) are cleared of air by opening and closing *f* and *l*. The flow of gas is stopped by disconnecting the polyethylene tubing (insert *A*) and closing the stopcocks *g* and *h* to the atmosphere. With the water level *e* in a position suitable for the proposed measurements, the initial flotation pressure of the diver is adjusted and read with the aid of the manometer *k*, *l*. Finally *d* is closed to the atmosphere. During the actual experiments—which may now be begun—gas exchange in the diver is read as the pressure changes ($\sim$ movements of the meniscus *e*) necessary to keep the diver afloat at a predetermined level.

shaft is pulled and from the other side when the tip is made. Thus the glass tapers excentrically into both tip and shaft, but the balance of the two is well adjusted and the diver will float upright (Fig. 2, VI). Personal experience is the only true guide in the choice of glass and in the method of pulling it to form a diver which can be made to float with a reasonable charge, i.e. one not essentially different from that illustrated in Fig. 2.

Calculation of CO_2 evolution.—The formula reported in earlier papers [16, 17] was used for calculating exchange of gases with low solubility, such as O_2. When measuring the evolution of CO_2 the amount of this gas dissolved in the liquid contents of the diver must be taken into consideration. Some uncertainty remains concerning the extent to which the liquid in the narrow channels in both ends of the diver—far removed from the gas space—serves as a solvent for the CO_2 formed during the experiments. This liquid volume, however, is very small. In the experiments presented in Fig. 4 it comprised less than 5 per cent of the liquid in the diver and less than 3 per cent of the total volume of the diver. The formula employed in this study for calculating CO_2 evolution was

$$\frac{CO_2}{t} = x\left(\frac{v \cdot V_G \cdot (B + h - e) \cdot 273}{V \cdot 10{,}300 \cdot (273 + t^\circ)} + \frac{v \cdot V_F \cdot \alpha CO_2 \cdot (B + h - e)}{V \cdot 10{,}300}\right)$$

in which x is the change in scale reading (mm per accepted time unit), v is the volume (μl) per mm burette and V is the volume of the gas phase of the "manometer" (μl); in our apparatus v/V was almost 1/200,000. V_G is the gas volume of the floating diver. B is the barometric pressure in mm H_2O (760 mm Hg ~ 10,300 mm H_2O), h is the initial equilibrium pressure of the diver (mm H_2O) read as the difference in height of the menisci on the double-branched manometer k (cf. Fig. 2, in which the initial pressure h is positive), e is the vapour tension of water at t° (mm H_2O), V_F is the volume of water in the diver (μl) and αCO_2 is the absorption coefficient for CO_2 at the temperature during the experiment.

Determination of V_F and V_G.—The volume of the liquid charge (V_F) is computed by subtracting the weight of the diver after oven-drying from the weight of the diver as soon as the actual experiment ends after washing in distilled water, removal of external liquid with a strip of filter paper and brief exposure to the air. The value thus arrived at is modified by the correction $V_G(h/B)$, to be added if the diver floats at positive pressure and subtracted if the diver floats at negative pressure. The volume of gas (V_G) in the floating diver is determined as follows. There is a constant ratio (f) between the weight of the glass used for the diver and the volume of air required to make the aggregate of glass and air buoyant in the medium. f is determined independently for the type of glass used. V_G is the dry weight of the diver multiplied by f.

RESULTS

Control experiments.—We are aware that our filling procedure (cf. stages III to V, Fig. 2) does not ensure initial saturation of the medium in the diver with 5 per cent CO_2 and 95 per cent N_2. It is possible for the pretreated medium

to lose to the atmosphere some of its CO_2 and N_2 while being poured into the dish (Fig. 2, III) or into the hollow slide (Fig. 2, IV) and to take up small amounts of O_2. When the diver has been filled, sealed and balanced to buoyancy, therefore, the gases will diffuse to equilibrium inside the diver. This process may last long, as the diffusion distances in the system are con-

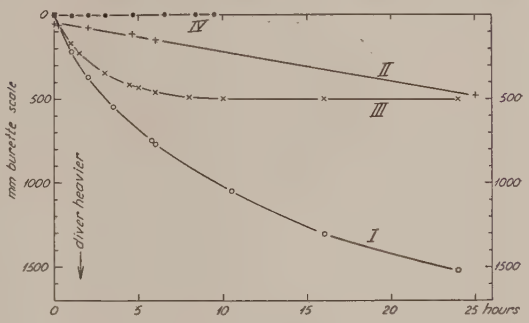

Fig. 3. The scale readings for freshly-pulled control divers charged with bicarbonate buffer solution (curve I) and with acetylthiocholine (AThCh)-bicarbonate solution (curve III). The preconditioning of the divers by prolonged internal contact with bicarbonate (curve II) or with AThCh-bicarbonate solution (curve IV) clearly eliminated the initial phase in which the divers rapidly become heavier.

siderable. With the same concentration gradient the amount of any one gas diffusing must vary directly with the absorption coefficient.

Because of its high α the diffusion of CO_2 can be expected to dominate over that of other gases. It should be possible to define filling procedure and diver dimensions so as to reduce to an acceptable minimum the time necessary for initial equilibration of the control diver. Our search for such conditions, however, led us to conclude that other sources of error play a dominant role. Glass, when suddenly exposed to CO_2-bicarbonate, seems to absorb CO_2 and to continue to do so for more than 24 hours. Fig. 3 shows the series of control experiments from which this inference was drawn. Curve I in this figure shows typical scale readings for a freshly-pulled control diver charged with 5 per cent CO_2 and 95 per cent N_2 and with bubbled bicarbonate buffer medium as described on page 570, but without AThCh. The flotation medium was the same as that in the diver charge. The manometer was perfused with gas as shown in Fig. 2. Curve I indicates that the diver became heavier with high initial rates. The system did not approach equilibrium for about 24 hours. This is much longer than we anticipated, judging from calculations based on formulas published by Linderstrøm-Lang [7, 8].

With a slight variation in procedure we obtained quite different results. We interrupted the diver fillings at stage III (Fig. 2) for 24 hours, during which time the filled ampulla was kept in air. We then blew the ampulla empty and refilled it, but this time concluding the procedure and setting the

diver afloat as described above. Curve II, Fig. 3 shows a typical result. Preconditioning of the glass by prolonged internal contact with CO_2-bicarbonate clearly eliminated the initial phase in which the diver rapidly becomes heavier.

When the diver, instead of being preconditioned, was filled as for the experiment shown in curve I, but with 6×10^{-3} *M* AThCh added to the buffer (inside and around the diver), the result was curve III. In our opinion the improvement as compared with curve I resulted from autohydrolysis of AThCh, with subsequent liberation of CO_2 wherever the CO_2 tension inside the diver was below that corresponding to 5 per cent (in gas of atmospheric pressure), and the pH thus higher than the stability limit of the substrate. This implies that the nonconditioned diver filled with CO_2-bicarbonate and in contact with this medium becomes progressively heavier because CO_2 is lost from the diver's gas phase. Since this loss does not occur by diffusion (cf. curves I and II), we suggest that the glass continues to adsorb or absorb CO_2 for twenty-four hours or more after it is brought into contact with CO_2-bicarbonate.

With the diver preconditioned by twelve hours' internal exposure to CO_2-bicarbonate with added AThCh, and then refilled as above described with AThCh-bicarbonate solution, curve IV is obtained. In five experiments it was established that after an initial period of about thirty minutes such divers showed no further significant change in equilibrium pressure. Over eight hours the scale readings were constant within $\pm$ 10 mm, or within a pressure

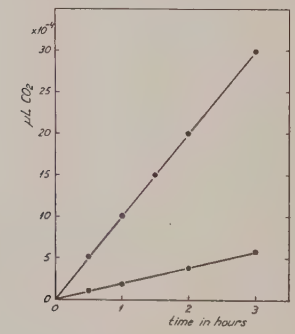

Fig. 4.

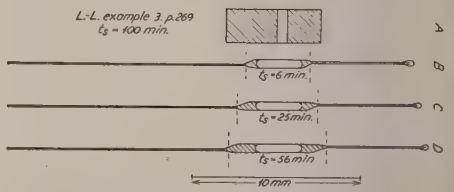

Fig. 5.

Fig. 4. Cholinesterase activity of a megakaryocyte (upper curve) and of a megakaryoblast (lower curve).

Fig. 5. Divers (*B*–*D*) charged in various ways. The duration (t_s) of the initial period in which reliable readings cannot be obtained is calculated by comparison with Linderstrøm-Lang's results ([9] p. 269).

range of less than 1 mm H_2O. During the 30-minute initial period, however, the equilibrium pressure in some divers showed variations up to ± 20 mm.

Cholinesterase experiments.—In the ChE experiments the divers were first filled with AThCh-bicarbonate solution plus gas, and the tips were sealed with wax. After about 12 hours the seals were broken off and the solution present in the tips was expelled. Fresh substrate-bicarbonate solution was sucked into the divers along with a cell, and stages V to VII of Fig. 2 were carried out.

ChE activity curves of a megakaryocyte (diameter 48 μ) and of a megakaryoblast (diameter 24 μ)—both from rat—are shown in Fig. 4. The experiments were conducted at 25°C. The megakaryocyte (upper curve) liberated from the bicarbonate buffer 100×10^{-5} μl CO_2 per hour, and the megakaryoblast (lower curve) 19.2×10^{-5} μl CO_2 per hour. These values correspond to the hydrolysis of about 0.013 and 0.0024 μg, respectively, of AThCh per hour.

The constants for the two divers were: $V_G = 08$ μl, $V_F = 0.44$ μl (upper curve), and $V_G = 0.54$ μl, $V_F = 0.24$ μl (lower curve).

COMMENTS

The initial period.—When the control diver, filled and treated as described for the typical experiment of Fig. 3, curve IV, is set afloat, we have frequently observed considerable (± 20 mm scale) changes of its equilibrium pressure. These initial changes, which usually involve less than the first half hour of the experiment, probably reflect the diffusion to equilibrium of the gases inside the diver. It is essential to point out that the time (t) required for diffusion to equilibrium of a finite amount of CO_2, introduced at time 0 into a defined location in the diver, is expected to be much longer than the time (t_s) which must elapse before constant rate of gas formation or gas uptake is reflected in linear changes in equilibrium pressure with time [7]. In experiments with a biologic object, t_s and not t is of interest. The purpose of the following evaluation is to give some idea of the dependence of t_s on the dimensions of the diver, a question of considerable practical importance.

According to Linderstrøm-Lang [7], t_s varies with b^2, b representing the length of the diffusion path in the liquid medium in the diver. The t_s value for CO_2 is little different from that for O_2. Linderstrøm-Lang [8] applied calculations to a diver system resembling the sketch A in Fig. 5. The diver is charged with two columns of liquid, 3 and 2 mm long (total volume 16 μl), separated by an air bubble (volume 2 μl). The respiring layer is situated in the smaller water volume, either adjacent to the air bubble or close to the impermeable membrane which forms the floor of the diver chamber. When the respiring sheet is adjacent to the air bubble the value of b which deter-

mines t_s was accepted to be close to the average length of the two columns ([8], p. 269). Our diver system closely resembles the system discussed by Linderstrøm-Lang. The actual shape of the charged diver may be any of those shown in Fig. 5. Lines have been drawn across the divers at distances of 0.5, 1.0 or 1.5 mm from the gas bubble, and the calculated values for t_s are reproduced. They are derived from the calculation that $t_s = 100$ minutes for $b = 2$ mm ([8], p. 247, table 4), accepting that t_s varies with b^2. The fact that the diver tapers off at both ends probably exaggerates the values given for t_s.

The situation, however, is somewhat complicated by the fact that the diver —in addition to the liquid between the lines in Fig. 5—carries small volumes of liquid in the long tail and tip. If the volume in these channels is not allowed to exceed 5 per cent of the total volume (air plus fluid) of the diver, its influence on the measurements can be disregarded, provided we do not claim more than about 5 per cent accuracy for the method. In any case it is probably difficult, for other reasons, to improve on this.

In our experiments the initial period required about 30 minutes, which is the time necessary for performing stages V, VI and VII of Fig. 2. The readings, however, were not commenced until 30 minutes after closing the stopcock d to the atmosphere (VII, Fig. 2), when the control divers were shown to have attained equilibrium.

Leakage of CO_2 from the diver.—The equilibrium pressure of the control diver remained constant within 1 mm H_2O (20 mm scale) for about 8 hours. This did not necessarily indicate that the diver was impermeable to CO_2. It may merely have reflected the establishment of complete equilibrium between the gases inside and around the diver. Introduction of the cell entailed a CO_2 concentration difference which increased as CO_2 formed in the diver in the course of the experiment.

With our apparatus the capacity for pressure adjustment without resetting of the instrument is exhausted when the CO_2 tension in the diver exceeds the tension in the flotation medium by 35 mm H_2O (initial equilibrium is assumed; length of burette—700 mm—corresponds to 35 mm water pressure). It is therefore of interest to calculate the maximum diffusion loss of CO_2, i.e. when the gradient is 35 mm H_2O. The formula employed is

$$\frac{\Delta CO_2}{h} = \frac{\varrho CO_2 \times A \times (p_1 - p_2)}{l \times B}$$

ϱCO_2 is the "standard rate of passage" of CO_2 in water. It is defined as μl

passing through a column of 1 mm², 1 mm long, in 1 hour, when the gradient is 1 atmosphere. Using Hüfner's values ([5], p. 407) we find that $\varrho CO_2 = 5.4$; $(p_1 - p_2)$ is 35 (mm H_2O); for B is introduced 10,300 (mm H_2O). The cross-sectional area (A) of the tail of the diver, through which pressure adjustment takes place, varies somewhat, but can easily be kept at less than 0.003 mm² (diameter 60 μ). As a rule the tail is longer than 15 mm, but not at its narrowest part. Hence we have inserted (10 mm) for l in the above formula. For $A = 0.003$ mm² we find $\Delta CO_2/h = 5 \times 10^{-6}$ μl.

In our standard diver a pressure change of 35 mm H_2O corresponds to the formation or consumption of 3.5×10^{-3} μl CO_2. If this amount of CO_2 were linearly produced over 1 hour, $(p_1 - p_2)$ would average 17.5 mm and the CO_2 loss by diffusion through the diver tail would amount to 0.7 ‰ of the simultaneous production in a diver with a tail 60 μ in diameter. But we seldom measure enzyme activities high enough to liberate CO_2 at that rate. With the production of CO_2 constant at, say, 3.5×10^{-4} μl per hour the experiment can be run for 10 hours before the CO_2 tension in the diver reaches the accepted maximum of 35 mm H_2O. The loss of CO_2—in percentage of the simultaneous CO_2 production—evidently increases linearly with time and attains a maximum by the end of the experiment. In the last hour it will be 1.4 per cent of the simultaneous CO_2 production in the diver in question. It should also be pointed out that for identical divers and for any fixed hour of the experiment the leakage of CO_2 represents a constant percentage error, irrespective of the rate of production.

We have not considered diffusion through the tip of the diver, assuming the solid wax to be impermeable to gases. Although there is some justification for this assumption ([15], sect. XII, p. 3), we may be mistaken. In that case CO_2 leaks from the diver about twice as fast as the calculated rate. In other respects, however, we have assumed more adverse conditions, i.e. favourable for diffusion. We feel justified in stating that the diver used can be made impermeable to CO_2 in the sense that leakage is negligible as compared with other sources of error in the measurements. The tip and tail of the standard diver should not be wider than 60 μ and the length of the narrow parts should exceed 10 mm.

Sensitivity and accuracy of the method.—The sensitivity of the method for absolute measurements is limited by the instability of the control diver. As reported in the foregoing, 5 runs with control divers showed that after an initial period of about 30 minutes the equilibrium pressure was stable within 1 mm H_2O for 8 hours. Stability is not substantially better over 1 hour than over many, as the scale readings vary rapidly within the 0.5 mm H_2O limit,

probably reflecting the offs and ons of the heater in the water bath. A pressure change of 10 mm H_2O (200 mm scale) accumulated in an experiment can thus be measured with 5 per cent accuracy. Accordingly, if 8 hours is the time stipulated to be the maximum duration of an experiment and the standard diver is used, half of the CO_2 being dissolved in the liquid, 10^{-4} μl CO_2/hour will be measured with $\pm$ 5 per cent accuracy. It is advisable, however, either to select the biologic object or to reduce the dimensions of the diver below those of the standard diver, so as to obtain more pronounced pressure changes. This will probably enhance the accuracy of the method.

The accuracy thus claimed for our diver technique of ChE determination is theoretical. We therefore checked the method by charging three divers with equal amounts of purified human plasma ChE. The error in charging the divers with this soluble plasma ChE was about 5 per cent. For diver 1: $V_G = 0.54$ μl, $V_F = 0.24$ μl; for diver 2: $V_G = 0.38$ μl, $V_F = 0.15$ μl; and for diver 3: $V_G = 0.4$ μl, $V_F = 0.12$ μl. The evolution of CO_2 in μl per hour was 22.1×10^{-5} in diver 1, 21.0×10^{-5} in diver 2, and 20.2×10^{-5} in diver 3. The experimental evidence thus supports the accuracy claimed for the method on the basis of theoretical considerations.[1]

SUMMARY

A Cartesian diver method is described for the determination of cholinesterase activity in single somatic cells (megakaryoblast, megakaryocyte). A megakaryoblast 24 μ in diameter was found to hydrolyze about 0.0024 μg, and a megakaryocyte measuring 48 μ, 0.013 μg of acetylthiocholine per hour. The accuracy of the method is discussed.

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[1] *Note added in proof.*—After the completion of the experiments presented in this paper, it was found that the observed absorption of carbon dioxide by the divers pulled from Jena glass did not take place in divers made from Phoenix glass. The use of Phoenix glass, therefore, eliminates the time-consuming preconditioning of the wall of the diver and makes it possible to introduce the cells immediately after the divers have been charged with the gas bubble and the substrate. The substrate used is 5×10^{-3} *M* acetylcholine chloride. The non-enzymatic hydrolysis of 0.1 μl of acetylcholine–bicarbonate solution (pH 7.4) was below the sensitivity of the present diver method.

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A GLUTAMIC ACID-GLYCINE MEDIUM FOR PROLONGED MAINTENANCE OF HIGH MITOTIC ACTIVITY IN GRASSHOPPER NEUROBLASTS[1, 2]

E. I. SHAW

Department of Zoology and Entomology, University of Tennessee, Knoxville, Tenn., and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A.[3]

Received June 7, 1956

The medium[4] employed heretofore for the culture of neuroblasts of the grasshopper embryo maintains mitotic activity at a reasonably high level for only 6–8 hours [8, 12]. This medium is a modified Ringer–Tyrode solution that was originally developed for the culture of tissues of the lower vertebrates. Ratios of sodium to potassium and of chlorides to organic anions in the fluids of the larval insect are very different from those in vertebrate body fluids [3]. The present study was undertaken, therefore, to determine whether changes could be made in Carlson's medium, which would reduce the stress exerted on the embryonic grasshopper tissues cultured in it, and consequently would maintain mitotic activity at a favorable level for long periods.

The method to be described was used to determine the ratios of the different cations present in the grasshopper egg. The content of 24 eggs of the grasshopper *Chortophaga viridifasciata* (De Geer) at a stage comparable to 14 days of development at 26°C was extracted in 3 ml of distilled water. The suspension was placed in a boiling water bath for 5 minutes to precipitate proteins. Chorions and denatured proteins were removed by centrifugation. The supernatant was shaken with 3 volumes of carbon tetrachloride to extract lipids and denature remaining proteins. One milliliter of the water layer contained the equivalent of all of the non-protein, non-lipid, water-soluble material of eight eggs. One-ml aliquots of these water extracts were digested with 1 ml of concentrated nitric acid heated to its boiling point in Kjeldahl flasks. Oxidation was completed by dropwise addition of 30 per cent hydro-

[1] Part of a thesis presented to the Faculty of the Graduate School of the University of Tennessee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology.

[2] Report prepared under Contract W-7405-eng-36 for the U.S. Atomic Energy Commission.

[3] Present address: Radiation Biophysics, University of Kansas, Lawrence, Kansas, U.S.A.

[4] Solution A: 7 per cent NaCl, 0.2 per cent KCl, 0.2 per cent $CaCl_2$, 0.1 per cent $MgCl_2$, 0.2 per cent NaH_2PO_4; Solution B: 0.12 per cent $NaHCO_3$; Solution C: 8 per cent dextrose. The ready-to-use medium is prepared by diluting 1 part of each of solutions A, B, and C with 7 parts of water [4, 9].

gen peroxide. The phosphorus concentration was determined by the method of Kitson and Mellon [13].

For flame spectrophotometric analysis of egg extracts, standard solutions were prepared of the nitrates of sodium, potassium, calcium, and magnesium and of phosphate as the ammonium salt. In standard solutions, the concentration of one ion was varied while that of the others was held constant. Emission versus concentration curves were determined at the wavelengths characteristic for each ion on the Beckman DU Flame Spectrophotometer. In the ranges of 0–160 parts per million (ppm) of potassium, 0 120 ppm of sodium, 0–400 ppm of calcium, and 0–400 ppm of magnesium, the changes in concentration of any one ion did not alter the accuracy of the determination of the concentration of the other ions. The concentration of ions was estimated through the measurement of the amount of emission obtained from extracts at the wavelength characteristic for each element and interpolation of this value on the curves prepared from standard solutions. The phosphorus content of the egg extracts was 0.8 mg per liter, a concentration that was found to be too low to interfere with the measurement of the other ions by flame spectrophotometry.

The flame spectrophotometric analysis of the water-soluble fraction of three egg extracts yielded weight ratios of potassium to sodium of 0.92, 0.95, and 0.98 for an average value of 0.95. On a molar basis, this is a ratio for sodium to potassium of 1.79 to 1.00. The molar ratio of sodium to potassium used in the new medium to be described was 1.66 to 1.00 with the substitution of the sodium and potassium salts of glutamic acid and glycine for the major portion of the sodium and potassium chlorides of Carlson's medium. Thus the concentrations of chlorides and organic anions are introduced in proportions comparable to those present in the blood or tissue fluids of larval insects [3]. The concentrations of calcium and magnesium were detectable but too low to determine accurately by the methods used. However, they were in a range that gave approximate ratios of sodium plus potassium to calcium and to magnesium that were similar to those in Ringer solution. The concentrations of calcium and magnesium, therefore, were kept the same in the new medium as in Carlson's medium.

The new medium is referred to as the GG medium to designate its two major components, glutamic acid and glycine. It is prepared from these stock solutions:

Solution A.—0.5 *M* potassium glutamate plus 0.5 *M* glycine (7.35 g of glutamic acid and 3.75 g glycine adjusted to pH 7.3 by the addition of concentrated

KOH free of bicarbonate, and diluted with pyrex-distilled water to make 100 ml of solution).

Solution B.—0.5 *M* sodium glutamate plus 0.5 *M* glycine (prepared in the same manner as solution *A* except that NaOH is used to adjust the pH).

Solution C.—$CaCl_2 \cdot 2H_2O$, 0.2 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; add pyrex-distilled water to make 100 ml of solution.

Solution D.—NaH_2PO_4, 0.8 g; $NaHCO_3$, 0.5 g; add pyrex-distilled water to make 100 ml of solution.

Solution E.—Anhydrous glucose, 7.0 g (or glucose $\cdot H_2O$, 7.7 g); add pyrex-distilled water to make 100 ml of solution.

The medium is prepared by mixing 0.75 ml of *A*, 1.25 ml of *B*, 1.0 ml each of *C*, *D*, and *E*, and approximately 3.0 to 5.0 ml of pyrex-distilled water, the exact quantity depending on the tonicity desired.

The importance of tonicity in studies of neuroblast mitotic activity cannot be overemphasized [10]. Slight changes in tonicity cause marked changes in mitotic activity. Approximately 3.3 ml of pyrex-distilled water was found to be the appropriate volume needed to dilute the stock solutions to prepare a medium with a tonicity suitable for dissection and for maintenance of the embryos in a relatively large volume of medium, the tonicity of which will not be affected by evaporation. During the preparation of the hanging-drop cultures, evaporation of water from the medium on the coverglass will cause a marked change in tonicity. For this reason the medium used for the preparation of the hanging-drop should be slightly more dilute than the medium in which the embryos are dissected. It should contain at least 0.8 ml of additional distilled water. The exact quantity must be determined by trial and error because the amount of evaporation will vary with the rapidity with which the operator can prepare the hanging-drop culture. If embryos are to be treated experimentally by the addition of solutes to the medium, adjustments in tonicity must be made for the osmotic activity of these solutes.

The technique of using isotonic medium for dissection and a more dilute medium for preparation of the hanging-drop cultures was compared with the technique of using a single concentration of medium for both operations. An analysis of variance of the results obtained from the two different techniques revealed that the variability between embryos was reduced markedly when the two-concentrations technique was used. The "least significant difference" may be reduced to about one-half by use of the two-concentrations technique.

The GG medium was designed so that further modifications could be made

easily. The ratio of sodium to potassium can be altered by changing the proportions of solutions *A* and *B*, as long as the total volume of these two solutions is kept at 2.0 ml in the ready-to-use medium. The pH of the medium is easily changed to any desired level by adjusting the pH of solutions *A* and *B* during their preparation. Since these are by far the most heavily buffered of the solutions, the pH of the medium is determined almost entirely by their pH.

Embryos of the grasshopper *Chortophaga viridifasciata* (De Geer) at an age equivalent to 14–15 days of development at 26°C were used to prepare hanging-drop cultures according to the method of Carlson and co-workers [4, 6, 7]. Slides, cover glasses, instruments, and solutions were sterilized with ultraviolet light by the method of Carlson, Hollaender, and Gaulden [9].

The number of mid-mitotic cells was counted at intervals of 22 minutes after the dissection of the egg and the explantation of the embryo into the culture medium. The mid-mitotic period (prometaphase, metaphase, and anaphase) in neuroblasts has an average duration of 22 minutes at 38°C in Carlson's medium [5]. If the duration of mid-mitosis is not altered by the treatment, each cell that passes through this phase will be counted only once. Thus the number of cells in mid-mitosis counted at 22-minute intervals is a valid estimate of the mitotic acitivity of the cultures. The duration of the mid-mitotic period in the GG medium was the same as in Carlson's medium and was unaltered by any of the modifications of the GG medium by the addition of amino acids. This indicates that, except under adverse conditions, the rate at which the neuroblast goes through mid-mitosis is within limits not a characteristic of the medium but is inherent in the cell. Cultures in Carlson's medium, as they age, progressively develop the characteristic appearance of cells immersed in a mildly hypertonic solution. The nuclear elements become more refractile and highly refractile bodies develop in the cytoplasm of dividing cells. These bodies can be caused to appear rapidly in newly prepared cultures by the use of strongly hypertonic medium. Cultures in the GG medium do develop the appearance characteristic of cells treated with mildly hypertonic solutions, but much more slowly than in Carlson's medium. If the refractile cytoplasmic bodies do appear soon after preparation of the cultures (which is infrequent), their appearance is transitory. They usually appear in old cultures in GG medium (36–48 hours after preparation) when the mitotic activity has fallen to a low level. Since the duration of mid-mitotic stages observed in individual cells is unchanged, and since the total number of dividing neuroblasts per embryo decreases with the age of the cultures in GG and Carlson's medium (Fig. 1), we can conclude that

fewer cells divide as the cultures age. Because the mitotic activity decreases more rapidly in Carlson's medium, one or several of the differences between Carlson's medium and the GG medium must be responsible for maintaining cell division for longer periods in the GG medium.

There are four major differences between the GG medium and Carlson's medium: (1) The chloride content has been reduced to about 5 per cent of the total anions, and glutamate and glycine are substituted for it. This conforms with the very low chloride content of the tissue fluids and blood of larval insects, which are mainly organic acids, the majority of which are amino acids [3]. (2) Since the flame spectrophotometric analysis of the egg yielded a molar ratio of sodium to potassium of 1.79 to 1.00, the molar ratio was lowered from approximately 45 to 1 in Carlson's medium to 1.66 to 1.00 in the GG medium. (3) The GG medium is more heavily buffered than Carlson's medium because of the substitution of the amino acids for the chlorides. This together with the slightly higher initial pH of the medium in the hanging-drop cultures provides a greater capacity for the neutralization of the carbon dioxide produced by the metabolism of the embryos.

Since Tipton and St. Amand [16] and Bodine and West [2] found that the addition of glucose with phosphate buffer did not affect the rate of respiration of grasshopper embryos, it is doubtful that the glucose of Carlson's medium is readily metabolized. Glucose was retained in the medium in order to avoid having the medium composed entirely of electrolytes. The dilution of the yolk components with the medium in the hanging-drop cultures suggests the necessity of introducing a sufficiently high concentration of oxidizable metabolites. Transaminating systems that can produce α-ketoglutarate from glutamate are present in cockroach muscle [1]. The presence of an active Krebs cycle capable of oxidizing α-ketoglutarate has been demonstrated in cockroach muscle [1] and in *Drosophila* larvae [15]. (4) Thus the glutamate in the GG medium, besides being an important anion and buffer, may also provide a source of energy not present in Carlson's medium.

The mixing of culture medium with the yolk routinely added to hanging-drop cultures dilutes the water-soluble components of the yolk. Their decrease in concentration through dilution may cause them to be limiting factors in cell division.[1] Additional quantities of cysteine, cystine, methionine, and ethanolamine phosphoric acid, in adjunct to the quantity contributed by the grasshopper yolk added to hanging-drop cultures, were tested for their

[1] Gaulden and Kokomoor [11] found that in Carlson's medium the absence of or the dilution of yolk below a certain concentration caused a decrease in mitotic activity. Preliminary studies with the GG medium indicate that the medium may support mitotic activity at the same level both in cultures prepared with and without yolk.

effect on the mitotic activity in cultures in GG medium. Medium containing added quantities of these naturally occurring components of grasshopper egg yolk [14] did not raise the level of mitotic activity or maintain it longer than in the unsupplemented GG medium. Also, none of these compounds

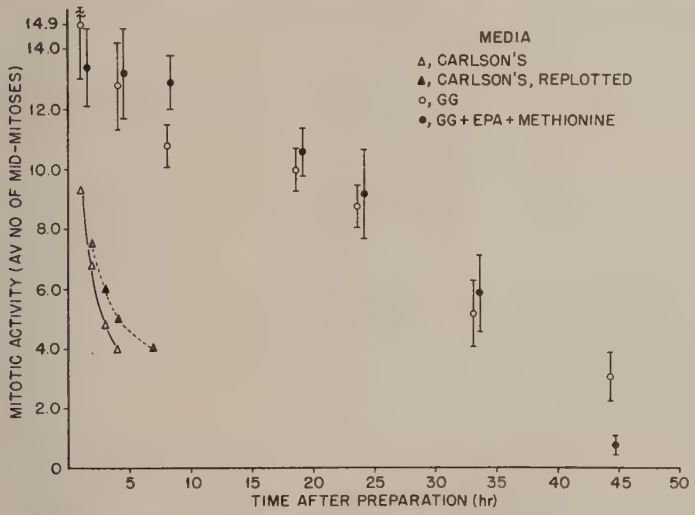

Fig. 1. A comparison of mitotic activity (average number of cells in mid-mitosis per embryo counted at 22 minute intervals) of cultures in Carlson's medium and in GG medium with time. The value for each point is the average of the data obtained from three successive observations on three embryos. Approximately 240 neuroblasts were observed in each embryo. The dashed line represents the data for Carlson's medium replotted from Carlson, Harrington, and Gaulden [6].

altered the duration of any of the mid-mitotic stages. The data for the experiment in which methionine and ethanolamine phosphoric acid were added together to the GG medium are plotted in Fig. 1. The addition of these two compounds, as with the other amino acids added singly, had no significant effect on the mitotic activity of neuroblast cultures. Thus the lack of effect of the additional amount of these compounds indicated that the dilution of the yolk by the culture medium did not cause these precursors in sulfur and methyl group metabolism to be limiting factors.

SUMMARY

1. A medium more compatible with grasshopper embryonic tissues than a medium previously used, was described. It has been designed easily to permit further modifications.

2. The ratios of sodium to potassium and of chlorides to organic anions are similar to those ratios found in the fluids of larval insects.

3. Mitotic activity of neuroblast cultures in the new medium is maintained at a usable level for 24–48 hours, a much longer period than previously possible.

4. The supplementation of the medium with the amino compounds cysteine, cystine, methionine, and ethanolamine phosphoric acid in addition to the amount provided by the yolk in the hanging-drop cultures, does not affect the level or duration of mitotic activity in neuroblast cultures.

I wish to express my gratitude to Dr. J. G. Carlson for his advice, criticism, and encouragement.

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BANDING IN SALAMANDER ERYTHROCYTES

A SHAPE CHANGE CORRESPONDING TO DISC-SPHERE TRANSFORMATION IN MAMMALIAN RED CELLS

W. D. TROTTER

Department of Human Anatomy, University of Oxford, England, and the Anatomy Department, University of Otago, New Zealand[1]

Received June 10, 1956

The phenomenon of banding was first observed by Barer during studies on the micro-spectrography of single salamander red cells when he noticed a non-specific notching of the absorption curve from the cytoplasmic portion of the cell [3]. Phase-contrast examination of cells showing this notching revealed that they were transversely striated (banded), and Barer [1] published a photomicrograph of cells showing this extraordinary appearance; but the phenomenon was not investigated further until the present work was begun at his suggestion. Although many early cytologists had studied living amphibian red cells [9], no record has been found in the literature of any appearance resembling banding.

Preliminary observations, described below, showed that the banded appearance was due to a change in cell-shape, the surfaces of the erythrocyte having become corrugated. They also suggested that the effect was due to conditions obtaining between the glass slide and coverslip. It is well known [11] that mammalian red cells mounted in saline between slide and coverslip, are transformed from discs to spheres, but Ponder [11] believed that no corresponding change occurs in nucleated erythrocytes. The purpose of the present paper is to describe the phenomenon of banding and the factors which produce it, and to show that it does appear to correspond to disc-sphere transformation in mammalian red cells.

According to Furchgott and Ponder [7] the latter phenomenon is due to (1) the adsorption on to the glass of an anti-sphering factor normally present in serum albumin, and (2) the diffusion of alkali from the glass to raise the pH of the medium to above 9.0. An investigation of the influence of these factors on banding suggested that this explanation was incomplete, and that a third factor, namely the presence of a lipoid substance, was important in

[1] This paper is based on work done in the Department of Human Anatomy at Oxford, while the author was on Study Leave from the University of Otago, Dunedin, New Zealand, to which he has now returned.

both banding and sphering. Slides and coverslips are usually contaminated with skin lipoid when cleaned with an ordinary cloth, but the lipoid can be removed by passing the glassware through a flame. A full account of the work on sphering has been published elsewhere [14], and the result of the present investigation will be discussed in the light of the interpretations given there; in fact, however, the work on banding actually preceded that on sphering.

The present study is really part of a more fundamental problem—that of the relationship of red-cell structure to red-cell shape, since the obvious question arises: why is it that the salamander erythrocyte which can sphere [13], remains a disc (although showing obvious changes), under conditions which cause mammalian red cells to become spherical? Meves [9] believed that amphibian erythrocytes differ from mammalian erythrocytes in possessing a strengthening ring around their margin; if so (and some observations will be cited in the present work to support the suggestion), this might explain the difference.

MATERIAL AND METHODS

All the blood used was from male specimens of *Salamandra maculosa*. They were kept for varying periods after reception and in some batches there was considerable mortality. However, the reactions of their erythrocytes did not appear to be affected, for whether from healthy or even moribund animals they all behaved similarly under similar conditions.

Red cells were obtained by snipping a small piece from the end of the tail and allowing the blood to drip into a vial containing 40–80 times its own volume of 0.8 per cent NaCl, which Gatenby and Beams [8] say is physiological for the salamander. The cells were allowed to settle by gravity, which they do rapidly because of their size; the supernatant fluid was then removed and replaced by more saline. The suspension was shaken and the process repeated; finally, sufficient supernatant was removed to leave a suspension containing a cell-concentration about 2–5 per cent of that of the original blood. Occasionally I used a modification of amphibian Ringer's solution,

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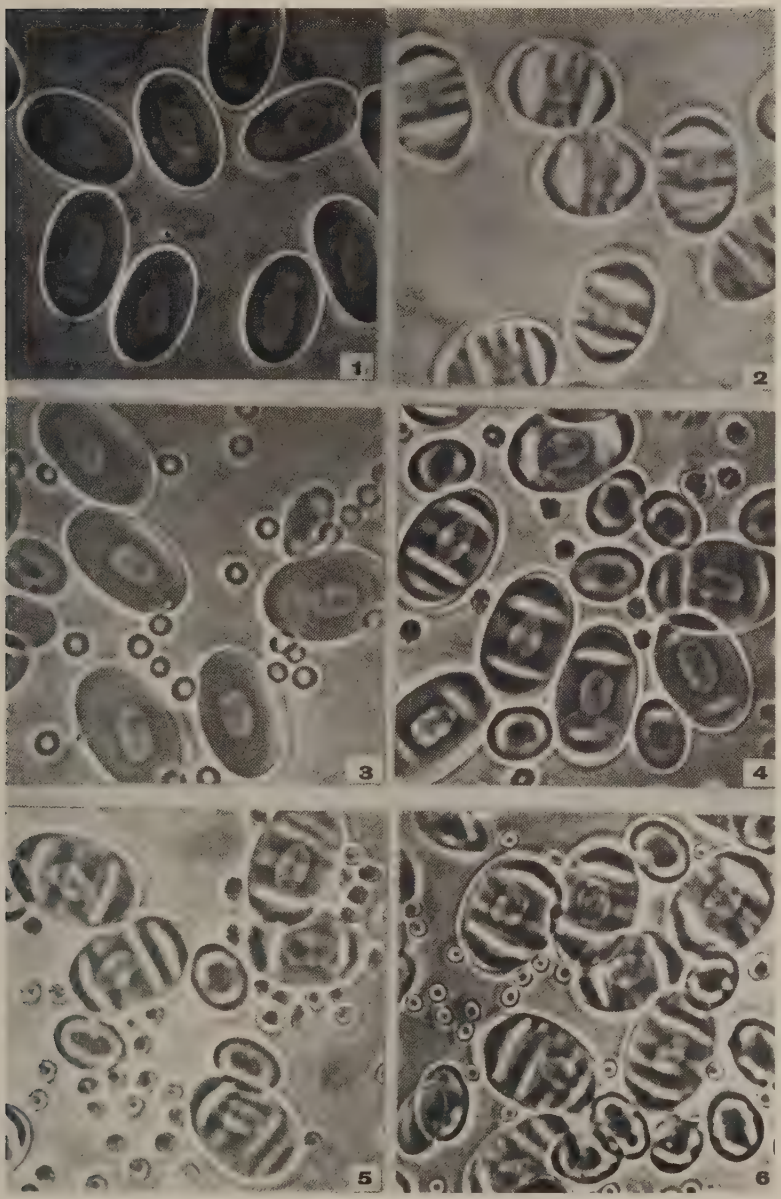
All photographs were taken by phase-contrast microscopy. The magnification is approximately 500 × in all figures. All figures are of cells suspended in 0.8 per cent NaCl.

Fig. 1. Salamander erythrocytes. Salamander serum added and cells mounted between new glass surfaces. Shows appearance of normal unbanded cells.
Fig. 2. Salamander erythrocytes. No serum present. Cells mounted between new glass surfaces to show typical field of banded cells.
Figs. 3–7. Correspondence between banding and disc-sphere transformation with varying pH. No serum present. *Figs. 3–6*. Triple suspensions of salamander, frog and human erythrocytes. *Fig. 7*. Salamander and human only. *Fig. 3*. pH 6.5 7.0. No banding, human cells perfect discs. *Fig. 4*. pH 7.0 7.5. Early banding and crenation. *Fig. 5*. pH 7.5 8.0. Moderate banding. *Fig. 6*. pH 8.5 9.0. Well-developed banding with finely crenated spheres. *Fig. 7*. pH 9.5–10.0. Advanced banding with perfect spheres.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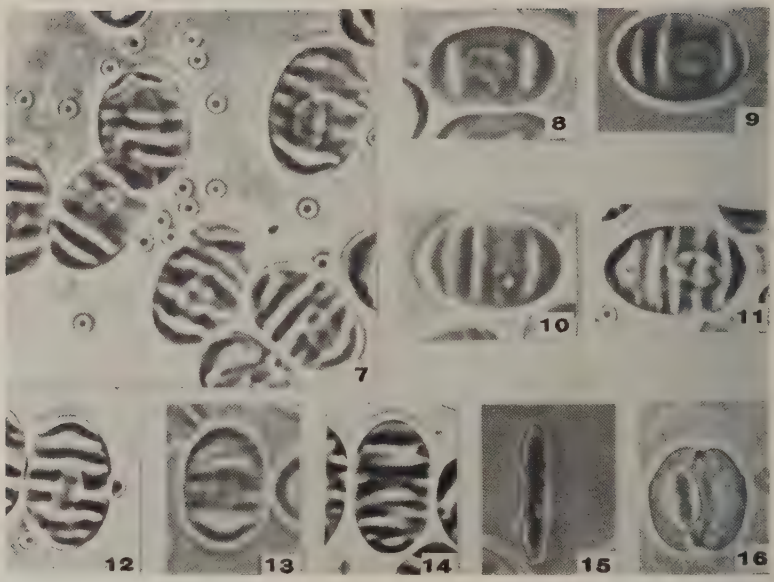

Figs. 8–14. Single salamander erythrocytes showing variations in the degree and form of banding.
Fig. 15. Profile view of banded cell to show surface corrugation.
Fig. 16. Salamander erythrocyte treated with *N*/20 HCl in 0.8 per cent NaCl. Surfaces have billowed out, but cell remains constricted around original rim. Marginal ring itself out of focus.

as described by Gatenby and Beams [8]. In this the NaCl was increased to 0.8 per cent (the concentration suitable for the salamander) and the other constituents (except the bicarbonate, which was omitted) were proportionately increased.

For the most part the cells were studied in slide-coverslip preparations; a drop or two of suspension was mixed on the slide with a drop of the reagent to be tested, and then covered. As the pipettes varied somewhat in calibre and the different reagents (e.g., albumin or lysin solutions) differed in surface tension, the size of the drops varied, and thus the concentrations given for slide-coverslip preparations made in this way are only approximate.

The pH was measured by means of the appropriate B.D.H. indicator solutions, for the particular range required. A drop of indicator was added to the fluid on the slide, after the coverslip had been removed at the end of microscopic observation. When glass slides and coverslips, both new and reclaimed, were used, and a drop of cell suspension, even a buffered one, was brought in contact with the surfaces, there were unpredictable changes in pH which made it impossible to adjust the pH of the bulk suspension beforehand so as to obtain a particular value in the slide-coverslip preparation at the end of the experiment. The pH was varied usually by adding to the drop of cell suspension a drop of dilute acid (*N*/200–*N*/500 HCl), or alkali (*N*/200–*N*/500 NaOH), or occasionally phosphate buffer solution. After the prepa-

rations had been examined for banding, the pH was determined merely from the colour assumed by the added indicator, because precise colour-matching with standard buffers would have been too difficult and time-consuming. The figures quoted, therefore, are only correct to about 1 pH unit.

Two preparations of bovine plasma-albumin were used—Armour's bovine plasma-albumin fraction V and the more highly purified Armour's crystalline preparation. Stock solutions, containing 1 per cent (1 g in 100 ml) of albumin, were made with 0.8 per cent NaCl as solvent, and were stored at 4°C, dilutions being prepared as required. Other proteins were also used —these will be described with the particular experiments.

Details of the preparation and use of various lysins employed will be mentioned when their effects are described.

The preparation of glass slides and coverslips has been described in a previous paper [14] and will not be discussed here. Most of the general observations on banding were made before the significance of glassware treatment was recognised and in these earlier experiments the new or acid-cleaned slides which were used must always have been contaminated with the lipoid sphering-factor.

The effect of various lysins was studied initially in deep chambers (25 × 10 × 0.5 mm) made by cementing coverslips on to a slide. This method was used to avoid the slide-coverslip changes, which do not occur or are very much delayed in deep chambers, where the layer of fluid between the glass surfaces is relatively thick [11].

To relate more precisely the degree of banding in salamander erythrocytes to that of disc-sphere transformation in mammalian red cells, suspensions containing both types of cell (in 0.8 per cent NaCl) were often used, a few drops of human blood from a finger-prick being added to the final suspension of salamander erythrocytes. Occasionally frog erythrocytes were added as well, and appear in some of the microphotographs, although the changes in these red cells are not considered here.

OBSERVATIONS

The Appearance of Banded Salamander Erythrocytes

The normal unbanded cell.—The unbanded cell (Figs. 1, 3) is slightly biconvex in profile, the broad surfaces showing a smooth regular curvature, and as seen by phase-contrast, is regularly elliptical. The surfaces of the disc appear smooth and homogeneous, and the cell as a whole appears grey in contrast with the background; the nucleus is fairly obvious, as it is usually of rather lighter contrast than the cytoplasm. It must be remembered that because of various optical effects [2], the contrast between nucleus and cytoplasm in a cell is not necessarily an accurate indication of the differences in their refractive indices, although the nucleus in a salamander erythrocyte may possibly contain less protein than the surrounding haemoglobin-rich cytoplasm. Unbanded cells of the type described may be seen in slide-coverslip preparations of whole blood, of saline suspensions containing serum or serum albumin, of suspensions whose pH is not above 7, and in suspen-

sions mounted between surfaces which have been freed from any trace of lipoid by flaming.

Banded cells.—The alternating light and dark striation of these cells (Figs. 2, 4–14) is in dramatic contrast to the homogeneous appearance of the normal erythrocyte. The nucleus, although still apparent, is somewhat obscured by the bands superimposed upon it. Even face on, there is a distinct impression of surface irregularity in banded cells, but this becomes quite definite when the cells are made to turn over in the fluid between slide and coverslip so that oblique and profile views showing a typical corrugated surface are obtained (Fig. 15). In many cells the corrugations on the two surfaces appear to correspond, i.e., convexities and concavities are directly opposite each other, but often the relationship was not so regular.

The type of banding varies considerably in different cells of the same and of different preparations. There are differences in the number of dark and light bands in any one cell (Figs. 8–14), in the degree of optical contrast between the bands (Figs. 11 13), in the sharpness of outline of individual bands (Figs. 12, 13), and in the length (Figs. 8, 9, 12, 14) and orientation of the bands, i.e., whether they are regularly parallel across the cell or appear crooked and even anastomose (Figs. 12–14). While some of these variations seem to be due to inherent differences between individual cells or between the cells of different animals, variations in the intensity of the shape disturbance naturally depend on variations in the pH, or albumin content, or on the presence of some banding reagent.

Variations in the degree of banding.—Because of this great variety of form, it is difficult to compare quantitatively with any accuracy the degree of banding seen in different preparations, but a broad classification can still be made. Thus banding may be slight or early as in preparations where the change is still progressing (Fig. 4), moderate (Fig. 5) or well-developed (advanced) (Figs. 2, 6, 7).

Where banding might be expected on theoretical grounds to be most advanced, i.e., in highly alkaline preparations with heavily contaminated slides, the cells show obvious surface distortion even in flat view, particularly under dark-ground illumination, the surfaces being crinkled rather than smoothly undulating. On the other hand, where banding is slight or even moderate, dark-ground study does not usually reveal more than the cell and nuclear outlines—no surface detail is apparent. Again, in advanced banding a greater proportion of corpuscles with crooked, anastomosing and incomplete bands is seen, giving the impression that the "skin" of the cells has become mechanically weaker and more easily crumpled.

While at one extreme we have the unbanded, uniformly grey cell, corresponding to the uncrenated mammalian disc (Fig. 3), at the other there is no well-defined end-form corresponding to the smooth-surfaced perfect sphere which is the culmination of the mammalian disc-sphere transformation (Fig. 7). Nevertheless, where salamander and mammalian erythrocytes are together in the same preparation the general intensity of shape disturbance of the former parallels the degree of disc-sphering (Figs. 3–7). But while it is true that the appearances of the cells are broadly comparable, there are certain differences in the degree of banding between the cells of different salamanders mounted together with the same human cells under apparently identical conditions. Thus the cells of one salamander might show moderate banding in a preparation in which the human cells were perfect spheres, while those of another salamander mounted with the same human cells would show advanced banding, the latter cells again being spherical. Again, in other preparations where the human cells were crenated discs, cells of one salamander might be moderately banded while those of another showed only slight banding. This suggests that the cells vary inherently in their response to conditions which cause banding.

The Conditions Influencing Banding

Initial observations.—Certain features were observed in salamander erythrocytes before it was discovered that banding was a phenomenon comparable with disc-sphering. These features are described here not only because they led to this discovery, but because they may be reproduced in slide-coverslip preparations whenever the glassware is not specially treated.

At first, I examined cell-suspensions using new glass slides and coverslips taken fresh from the box. A drop of cell-suspension was placed on the slide and the coverslip made to fall with its centre approximately over the drop. The fluid spread readily beneath the coverslip, but the cells were found to be unevenly distributed; while the majority remained fairly closely packed at and around the original drop site, others, carried further with the spreading fluid, were scattered more thinly between the drop site and the edges of the coverslip. Banding was most advanced among the most peripheral cells. Although on new slides this banding was well-developed probably even before the preparation could be examined and before it seemed likely that marked evaporation could have occurred, the tonicity of the suspending fluid was varied in order to find out if this was a significant factor. Alterations in the strength of the saline solution from 0.5 to 0.9 per cent, however, made no difference to the degree of banding, although above this range there was

distortion of the bands, in the form of an irregular crinkling of the cell surface, while below it haemolysis set in. Even sealing the edge of the coverslip with vaseline did not reduce the intensification of banding with time in the peripheral cells (in preparations containing 0.5 per cent NaCl). Moderate changes in tonicity therefore did not seem to be an important factor in the genesis of banding.

Sometimes (inadvertently at first) the coverslip was allowed to fall eccentrically on the drop of suspension; in such cases the closely-packed cells, which had moved least between the glass surfaces, were now found near one edge, while the thinly scattered ones which showed marked banding had moved only to the centre of the coverslip. This finding confirmed the impression that the extent to which the cells had travelled or the closeness of the packing were factors concerned in banding, the former favouring, the latter hindering it.

On acid-cleaned reclaimed slides, which liberate much less alkali, the results were the same, although because of the lower pH, banding developed more slowly and was never so well marked as with new glass. It had often not begun when these preparations were first examined, so that the phenomenon was obviously not present in the bulk suspension, but only developed as a consequence of mounting the cells between the slide and the coverslip. These observations pointed clearly to the similarity between banding in salamander erythrocytes and slide-coverslip disc-sphering in mammalian red cells.

One reason for the greatest banding among the cells which spread furthest (especially on new slides) was discovered by placing a drop of phenolphthalein solution on a new slide and covering as for a drop of cell suspension. The fluid around the margins of the spread drop became pink, indicating a pH of 10–11, while that near the centre remained colourless; hence banding is apparently associated with the higher pH at the periphery. Factors additional to pH variations may also contribute to the differences in banding between centre and periphery. The peripheral cells had each travelled across a greater surface of glass than the central ones, which would facilitate adsorption of albumin from cell surface to glass. Conversely, the albumin concentration where cells were closely packed might be sufficient to saturate the adsorbing power of the glass, before enough had been removed from the cell surfaces to permit banding. All these considerations lent support to the hypothesis that banding and disc-sphering were comparable.

Variation in the degree of disc-sphering of mammalian red cells is also seen in different parts of a slide-coverslip preparation, but the effects here are less pronounced because these cells move and scatter more freely in the spreading fluid than do the very much larger salamander erythrocytes.

The Correspondence between Conditions Causing Banding and Disc-Sphering

In many of the experiments about to be described mixed suspensions of human and salamander erythrocytes were used to give a simultaneous and direct comparison of the influence of various factors on both kinds of cell.

The effect of pH.—However the final pH was reached, whether by added acid or alkali, or by the effect of alkali from the glass itself, the degree of banding could be correlated with the pH. Thus below pH 7 no banding occurred and human cells in the combined suspensions remained perfect discs. When excess acid was added to produce a very low pH, an irregular crumpling of the salamander cells occurred (the human cells in the same preparation often became cupped), but this was quite different from banding. In alkaline preparations above pH 9 the salamander cells showed typical advanced banding and the human cells became uncrenated spheres (Fig. 7). At pH levels between 8 and 9 moderately banded salamander erythrocytes and finely crenated spheroids and spheres were seen (Figs. 5, 6); while between pH 7 and 8 slight to moderate banding correlated with coarsely crenated discs and spheroids (Fig. 4). In very alkaline preparations (NaOH of the order of $N/600$ or stronger) the highly-banded cells showed, after some minutes, obvious and severe shrinkage and collapse leading ultimately to cytolysis.

The effect of plasma albumin.—Banding was completely prevented by the addition of salamander plasma, frog plasma, human plasma, horse serum, and by various purified preparations of bovine and human plasma albumin. Purified bovine plasma albumin gave complete inhibition of banding at concentrations of 0.5 per cent in the final preparation, and marked inhibition at concentrations down to 0.1 per cent; it showed an appreciable inhibiting effect at concentrations as low as 0.02 per cent. Even with more dilute solutions, in which banding did occur, it developed more slowly, involved fewer cells and was less advanced even in the most affected cells, than in control preparations. Because of the difficulty of distinguishing slight differences in banding in different preparations, it was not possible to determine the lowest concentration at which albumin acts to inhibit banding. With mammalian erythrocytes, however, the end points of perfect sphere and disc are well-defined, making quantitative work possible [7].

The effective concentrations of the various plasmas used were not estimated in detail, but where the albumin content of the plasma was approximately equivalent to that of solutions of purified albumin the effects were similar.

What has been described for the purified albumin experiments applies also to those with plasma.

While all plasma and serum preparations, whatever the source, inhibited banding in glass slide-coverslip preparations, other proteins which were tried (egg albumin, 2 per cent; gelatin, 0.5 per cent; peptone, 5 per cent; and crude human haemoglobin, 10 per cent approximately) were found to have no inhibitory effect. In mammalian disc-sphering, only blood albumin is effective and again it does not matter from what animal it is obtained.[1]

Not only does plasma albumin prevent banding, but when added to the fluid of a slide-coverslip preparation in which the cells are already banded, i.e., by lifting the coverslip and mixing a drop of albumin solution with the fluid, the erythrocytes revert to the smooth homogeneous unbanded state.

The effect of added oleic acid.—Because Furchgott [5] had found that some skin lipoid or oleic acid, when added to bulk suspensions of mammalian erythrocytes, appeared to act as antagonists of the anti-sphering albumin, the effect of adding oleic acid to preparations of salamander erythrocytes was tried. In some experiments direct mixtures of oleic acid in 0.8 per cent NaCl were added to the cell suspensions, but usually a methanol solution of oleic acid was made first and appropriately diluted with saline. Control experiments showed that methanol itself, in the concentrations in which it finally came into contact with the red cells (even as high as 1 per cent), had no visible effect. Cell suspensions containing added oleic acid were examined in deep chamber preparations. Under such circumstances marked banding occurred; controls, namely, salamander red cells in saline media without oleic acid, remained unbanded in deep chambers, except for a few cells which came into intimate contact with parts of the glass which were not easily freed from contaminants. Advanced banding occurred in 2–3 minutes even with very low concentrations of the order of 1 part of oleic acid and 10 of methanol in 200,000 of saline, while a tenth of this concentration produced banding in about 10 minutes. Oleic acid may also cause banding even in the presence of added albumin, for 1 part in 3000 of oleic acid is effective in the presence of 1/300 bovine plasma albumin. At lower concentrations, however, the results in the presence of albumin were rather capricious, and no explanation has been found for their variability. Whether the oleic acid which caused banding in very low concentrations in the absence of added albumin did so by antagonising the albumin adsorbed on the cell surface and allowing the pH of the medium to have its effect, as suggested by Furchgott [5] in the

[1] Barer and Gaffney (*Nature* **177**, 277 (1956)) have recently reported that the plasma of certain rodents may contain a sphering factor which opposes the antisphering activity of plasma albumin.

case of mammalian erythrocytes, or whether it acted like some haemolytic agents which cause sphering could not be decided from these experiments. In favour of the latter view was the observation that with higher concentrations of oleic acid, the banded cells after some minutes lost their bands and underwent peculiar changes in shape (to be described in another paper) leading eventually to haemolysis. Whatever the mechanism, it was plain that oleic acid could cause banding in deep chamber preparations of salamander red cells just as it produces sphering in mammalian erythrocytes.

The effect of various haemolysins on banding.—Mammalian red cells may be converted from disc to sphere by the addition of small (sublytic) quantities of a number of haemolysins [11] and the process can be inhibited or reversed by adding plasma albumin, or by washing away the haemolysin. The effect of adding small quantities of lysins to deep chamber preparations of salamander cells was studied to see whether the nucleated cells would respond by developing bands corresponding to the crenation and sphering of mammalian cells with these reagents.

Although no detailed quantitative study was made, the following lysins were used in approximately these concentrations in the final preparation: 1/3000 sodium taurocholate; 1/100,000 saponin; 1/300,000 digitonin; 1/80,000 sodium dodecyl sulphate. These dilutions were freshly prepared from stock solutions in 0.8 per cent NaCl of 1 per cent sodium taurocholate, and 0.1 per cent in all the others; the stock solutions were stored at 4°C. All these substances were found to produce banding in deep chambers in contrast to controls without added lysin. Lecithin in a watery mixture of unknown but probably high concentration was similarly effective. Plasma albumin in a concentration of 1/300 inhibited the development of banding in the presence of these lysins. The influence of various other substances known to inhibit lecithin sphering of mammalian erythrocytes [11] was not tried, as the quantitative conditions have to be carefully adjusted to study such effects. Nevertheless, the experiments demonstrate quite clearly that banding is produced in salamander erythrocytes by reagents which produce crenation and sphering in mammalian red cells.

Banding on non-glassy surfaces and the importance of lipoid contamination.—The behaviour of salamander red cells between surfaces other than glass was investigated, because it had been suggested in the literature [14] that factors other than alkalinity and adsorption of albumin might be concerned. Initially, only old reclaimed quartz and mica materials were available and between these surfaces banding took place just as with glass, its degree varying with the pH of the suspension medium. A few new mica and plastic slides

and coverslips were obtained; with these it was found that, even where the pH was sufficiently alkaline to allow marked banding, it did not occur when they were new, but when they had been washed and rubbed dry with a soft cloth, banding developed, its extent varying with the pH of the fluid.

This observation led directly to the discovery that even the most minute traces of contaminating lipoid are of vital importance in slide-coverslip disc-sphering and banding. Most of the exploratory work on this was done with mammalian cells, but a few experiments with salamander erythrocytes confirmed the fact that banding never occurs between glass surfaces which have been freed from lipoid by flaming, boiling or washing in pure ether. When slides and coverslips decontaminated in this way are, before use, rubbed with a soft cloth which has been handled in the ordinary way, they become recontaminated with traces of skin lipoid from the cloth and again acquire the ability to produce banding if the pH is suitable. Just as in disc-sphering [14], the deposition of a minute quantity of oleic acid, stearic acid or skin lipoid by the evaporation of a drop of ethereal solution placed on a flamed slide permits banding over the area thus contaminated. While this is consistent with the already observed ability of oleic acid to cause banding in deep chamber preparations, these experiments establish particularly the role of lipoids, such as oleic acid, as actively banding, rather than simply "anti-anti-banding factors".

Evidence that Banding Represents a Tendency to Sphering Resisted by Cell Structure

In the disc-sphere transformation of mammalian red cells no appreciable change in cell volume accompanies the change in shape [11]. Thus the ratio of surface area to volume must be decreased. The crenations of the intermediate stages represent presumably a folding of the now rebundant surface material of the cell, although what becomes of this excess "skin" in the finally smooth sphere is still a mystery [11].

Although the salamander cell remains a disc, while the mammalian cell becomes spherical, it is possible that the surface corrugation of the former might result from a tendency to sphering—a tendency, however, which is unable to overcome the resistance of those forces determining the normal disc shape. Such a tendency, if it exists, should surely express itself in the cell gaining a more circular outline. This possibility was, therefore, explored.

The greatest length and breadth of 55 well banded and the same number of unbanded cells selected at random in preparations from the same animal, were measured with an eyepiece micrometer. The mean length/breadth

ratio of banded cells was 1.413 (S.D. 0.114) and of unbanded cells 1.527 (S.D. 0.147), the difference between these ratios being significant ($P < 0.01$). Therefore the banded cells were slightly shorter and broader than the unbanded. Although in the absence of volume measurements it is impossible to be sure, the figures suggest that the corrugation of banding represents a folding of redundant surface "skin" as the banded cells, albeit still discs, acquire a slightly more circular outline, i.e., approach more closely to a sphere than the unbanded.

Under the influence of acids ($N/20$ HCl in the final medium) salamander erythrocytes undergo considerable swelling. When this happens, however, the original rim seems to swell much less than the rest, so that the cell appears as if it were constricted by a rigid ring (Fig. 16). Other phenomena suggesting that such a ring exists in amphibian erythrocytes will be described in a later paper; meanwhile it can be said that my observations provisionally confirm those of Meves [9] who, by using acids, was able to demonstrate the presence of such a ring. This might well be the reason why salamander red cells remain as discs while mammalian erythrocytes become spheres.

DISCUSSION

At first sight the transverse banding seen by phase-contrast might appear to be due to alternating changes in the refractive index of the cells. Pulvertaft [12] interpreted in this way a light-dark zoning which he saw in avian erythrocytes, although from a personal examination of similar material, I believe that a change in shape is responsible. In banded salamander red cells, from the non-specific nature of the notching in the light absorption curve, it appears to be more likely that a shape change, namely, the corrugations, is responsible, than that there has been an alteration of the cell sufficient to produce marked local variations in the refractive index. However, this view demands further consideration.

The thickness of a salamander erythrocyte near its edge is at least 2 μ (i.e., 4 wavelengths of visible light). I have estimated the refractive index of salamander erythrocytes by the method described by Barer, Ross and Tkaczyk [4] and find it to be approximately 1.40. Thus the phase change through the cell would be about $0.28\ \lambda = [1.40 - 1.33$ (refractive index of medium)$] \times 4\ \lambda$. In a banded cell, to use a visual estimate based on Fig. 15, the thickness of cell between the troughs and crests of the corrugations might vary from 1.5 to 2.5 μ, giving phase changes of 0.21 λ or 0.35 λ respectively. These differences are really quite large since the sensitivity of phase-contrast

is sufficient to detect phase differences of $\lambda/100$ or less, so marked variations in intensity should result from the difference of 0.07 λ assumed to be present here. The absolute phase change of 0.28 λ for a 2 μ-thick erythrocyte is sufficient to produce reversal of contrast under some conditions, so that some 'light' bands may even appear brighter than the general background. Finally, the halo effect in phase-contrast images might well intensify the contrast between neighbouring strips of the banded salamander red cell.

That the phenomenon of banding occurs under just those conditions which produce disc-sphere transformation in mammalian red cells is evident from the numerous observations cited above. My own experiments have been only roughly quantitative and if more delicate methods had been applied it might have been found that there were differences in detail (e.g., in pH requirements or in other factors) between salamander and human erythrocytes.

What is more important is to decide whether or not the shape change shown by the salamander erythrocyte is the physico-chemical equivalent of crenation and sphering in the mammalian red cell. If one insists on the criterion of the cell actually becoming spherical as Ponder [10]—to judge from his conclusions—apparently does, then such changes in appearance as are seen in salamander and in other nucleated red cells cannot be regarded as *corresponding* in the strict sense to disc-sphere transformation. Until we know the precise chemical and physical interactions responsible both for disc-sphering and for banding, the problem is largely a matter of definitions. Yet as it is likely that in their general composition all erythrocytes contain similar substances, it is not unreasonable to expect that these materials will react in the same way when the cells are subjected to identical experimental conditions.

In both banding and disc-sphering, what is seen microscopically is a bending or folding of the surface layers of the cell. In the large salamander erythrocyte the appearances convey the strong impression that this bending is associated with a decrease in the mechanical strength of the surface material of the red cell. Alternatively, the physico-chemical changes which produce banding might act, not on the surface layers directly, but on other cell components. The corrugation and folding might thus follow passively an increase in the forces tending to make the cell spherical. At present it is impossible to decide which components of the cell are involved.

In the mammalian erythrocyte the surface layers during crenation seem to behave like a solid "skin", though in the final state the spherical cell appears as a liquid droplet. It is even possible that some local as opposed to general

molecular grouping, which is microscopically invisible, has broken down. Furchgott's [6] observation that the crenations can be made to disappear and reappear at the same place in a single mammalian red cell suggests either pre-existing local differentiations determining the pattern of crenation, or persistent local weaknesses following the first crenation. The usual tendency, especially among those interested in the chemical physiology of the erythrocyte is to regard it as an essentially homogeneous structure. The very elaboration of molecular models of the erythrocyte (or its "membrane")—models which can only display the tiniest fraction of a single erythrocyte—underlines the assumption that the whole cell is merely a repetitive aggregation of such units. Yet, some who have studied the microscopic behaviour of erythrocytes under experimental conditions tend, on the other hand, to postulate structural heterogeneity—for instance, the rim of the cell may be differently constructed from the biconcavity, or even one small part differently from a small part elsewhere.

It is in connection with these two approaches to red cell structure that the phenomenon of banding would appear to have general significance. The salamander erythrocyte clearly reacts to the same conditions which cause a shape change in the mammalian red cell, but it does not become spherical. The simplest explanation of this failure would be that the rim of the cell is composed of material which is more inflexible and will not accordingly yield to the changed physico-chemical environment sufficiently to permit sphering. The reduced length/breadth ratio of banded cells is suggestive evidence that the elliptical erythrocyte is tending to become spherical when it undergoes banding. Meves [9] in a paper which modern writers seem to have neglected or misinterpreted (or perhaps even mistrusted, since so many claims to have demonstrated local structure in erythrocytes have been based on artefact) has given excellent illustrations of a mechanically stronger ring ("Randreife") at the periphery of the amphibian red cell. I believe that I have been able to confirm this finding (Fig. 16), but this matter will be discussed in a separate paper.

Ponder [10] has described briefly the changes which he saw in various nucleated erythrocytes under conditions causing disc-sphering. In general I have been able to confirm his observations, though considerable amplification is necessary. This subject also will be dealt with in a separate publication. It suffices to say at present that the appearances were different from banding, though also essentially due to changes in the surface contours of the cells. One cannot rest content merely with the observation that different species act differently. The fundamental problem is: whether it is possible

to correlate the visible behaviour of erythrocytes of different species under experimental conditions, with precise differences in the construction of the cells. Although this was a goal, almost impossible of attainment by the earlier workers, the invention of the phase contrast microscope, the improvement of electron microscopy of cytological material, and the greatly increased biochemical knowledge of the red cell have provided fresh tools with which to renew the attack.

SUMMARY

1. Salamander erythrocytes under certain conditions show by phase contrast microscopy an alternately light and dark transverse striation or "banding".

2. The detailed form of "banded" cells is described, and the phase contrast appearance is considered to be due to variations in the thickness of the cell consequent upon a corrugation of the cell surfaces.

3. The conditions producing banding are very similar to those causing disc-sphere transformation in mammalian red cells, especially the disc-sphere transformation between slide and coverslip.

4. Banding occurs: (*a*) when the cells are mounted in a saline medium, which is free of plasma albumin and of alkaline pH provided the surfaces of the slide or coverslip (or the medium itself) are contaminated with a lipoid "banding" (or sphering) factor; (*b*) in plasma-albumin-free medium containing small (usually sublytic) quantities of various haemolysins.

5. The significance of banding in the general problem of red cell structure is discussed.

I wish to thank Dr. R. Barer for his valuable advice and criticism; Professor Sir Wilfrid Le Gros Clark for his interest and for providing facilities in his department; Professor W. E. Adams for his most helpful criticism of the manuscript; Messrs. R. Underwood and J. G. Howard for their help with the photomicrographs, and Miss M. E. Ogilvie and Mrs. M. M. Miller for mounting the illustrations and typing. This work was made possible by grants to Dr. Barer from the Nuffield Foundation and Rockefeller Foundation.

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ALLEVIATION OF THE ANTIMITOTIC EFFECT DUE TO CERTAIN MERCAPTOACETIC DERIVATIVES OF 1:4-NAPHTHOQUINONE BY METHIONINE

E. FRIEDMANN and I. SIMON-REUSS

Department of Radiotherapeutics, University of Cambridge, England

Received June 10, 1956

Mercaptoacetic derivatives of 1:4-naphthoquinone are inhibitors of mitosis in tissue cultures of chick fibroblasts. The monomercaptoacetic derivative (quo-*mo*) exerts a mitotic inhibition of 72 per cent at 10^{-5} *M*, the 2-hydroxy-3-mercaptoacetic derivative (hydroxy-quo-*mo*) inhibits by 87 per cent at 10^{-7} *M* and the *bis*-mercaptoacetic derivative (quo-*bis*) by 39 per cent at 10^{-7} *M* [11].

Attempts to use these inhibitors of mitosis for retardating the growth of malignant cells in animals were unsuccessful. Neither the growth of the Walker carcinoma of the rat, nor the development of the rat Jensen sarcoma or of the mouse Crocker sarcoma 180 could be influenced by applying these substances. An investigation has therefore been begun to look for reactions which may lead to an explanation of these discrepancies.

The substances produced by the interaction of mercaptoacetic acid with quinones are thioethers. The physiological action of some thioethers can be antagonised by other thioethers. Thus L-methionine and D-methionine alleviate the growth inhibition caused by L-ethionine, D-ethionine or DL-ethionine in rats maintained on 25 per cent casein diet [17]. Ethionine produced toxic reactions when given to human beings, and its effect could be alleviated by methionine [5]. Transplanted fibrosarcoma and the Jensen sarcoma in rats have a low methionine content, and the growth of these tumours could be inhibited by DL-ethionine [14].

These results suggested the investigation of methionine as an alleviator of thioethers such as the mercaptoacetic acid derivatives of quinones.

METHODS

The experiments were done on tissue cultures of chick fibroblasts, and the methods used have been described repeatedly [10, 15]. The substances were tested as sodium salts. Methionine was applied to tissue cultures of chick fibroblasts as DL-methionine,

L-methionine and D-methionine. The preparations were supplied by L. Light & Co. Ltd.

L-methionine and D-methionine are inhibitors of mitosis as well as the mercaptoacetic adducts of 1:4-naphthoquinone. Each experiment therefore comprised 4 series of cultures: controls, cultures treated by methionine, by the mercaptoacetic adduct, and by a mixture of methionine and the same mercaptoacetic adduct. Every figure given in this paper is the average obtained in six tissue cultures evaluated statistically. In all the tables the different phase distributions are expressed as percentages of the total mitotic counts.

The influence of methionine on mitotic inhibition had to be treated separately from its influence on the changes of phase distribution and chromosomal abnormalities, as in the systems investigated these processes do not run parallel.

As in the previous paper, quo-*mo* stands for the *mono*-mercaptoacetic derivative of 1:4-naphthoquinone, hydroxy-quo-*mo* for quo-*mo*, substituted in vicinal position with a hydroxylic group, quo-*bis* for the *bis*-mercaptoacetic derivative of 1:4-naphthoquinone and B-*tetra* for the *tetra*-mercaptoacetic derivative of 1:4-benzoquinone.

RESULTS

DL-*Methionine.*—DL-Methionine was tested in 10^{-4} *M* concentration on quo-*bis* applied at 10^{-7} *M* to tissue cultures. At this concentration quo-*bis* causes a large accumulation of cells in metaphase, but inhibition of mitosis is not regularly observed.

Fig. 1 gives the phase distribution after applying DL-methionine on quo-*bis* at the concentration mentioned above.

Under the influence of DL-methionine the accumulation of cells in metaphase, produced by quo-*bis* at 10^{-7} *M*, is considerably diminished. The phase distribution is nearly normal but clumped chromosomes and binucleated telophases are to be found. A slight effect on resting cells is apparent.

L-*Methionine.*—L-Methionine has been investigated in the systems L-methionine 10^{-4} *M* / quo-*bis* 10^{-6} *M*, L-methionine 10^{-5} *M* / quo-*bis* 10^{-6} *M*, and L-methionine 10^{-5} *M* / quo-*bis* 10^{-7} *M*. Table I shows the influence of L-methionine on the mitotic inhibition caused by quo-*bis*.

In the system L-methionine 10^{-4} *M* / quo-*bis* 10^{-6} *M* complete alleviation

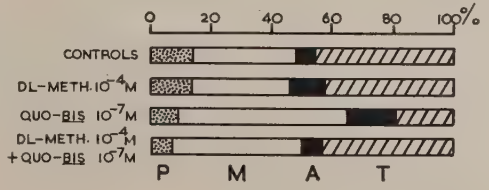

Fig. 1. Phase distribution after application of DL-methionine 10^{-4} *M* on quo-*bis* 10^{-7} *M* in tissue cultures. (*P*, *M*, *A* and *T* stand here, and in the following figures, for prophase, metaphase, anaphase and telophase respectively.)

TABLE I

Influence of L-methionine on the mitotic inhibition caused by quo-*bis*.

Substance applied	Mitotic inhibition as per cent of the mitoses in the controls		Substance applied
I L-Methionine 10^{-4} *M*	49 (± 3.1)	13.5 (± 2.2)	I L-Methionine 10^{-5} *M*
II Quo-*bis* 10^{-6} *M*	45 (± 4.7)	54 (± 2.7)	II Quo-*bis* 10^{-6} *M*
I + II	nil	21 (± 2.5)	I + II

of the mitotic inhibition is observed. In the system L-methionine 10^{-5} *M* / quo-*bis* 10^{-6} *M* the mitotic inhibition is considerably decreased.

The corresponding phase distribution is given in Fig. 2.

The accumulation of cells in metaphase caused by quo-*bis* and by L-methionine is diminished in the system L-methionine 10^{-4} *M* / quo-*bis* 10^{-6} *M* In the system L-methionine 10^{-5} *M* / quo-*bis* 10^{-6} *M*, L-methionine 10^{-5} *M* alone does not affect the phase distribution, but the arrest in metaphase caused by quo-*bis* 10^{-6} *M* is decreased by adding L-methionine 10^{-5} *M*. In the system L-methionine 10^{-5} *M* / quo-*bis* 10^{-7} *M*, L-methionine has failed to diminish the accumulation in metaphase produced by the quo-*bis*. The normal phase ratio is not completely restored by L-methionine in any of the experiments and even in the system L-methionine 10^{-4} *M* / quo-*bis* 10^{-6} *M*,

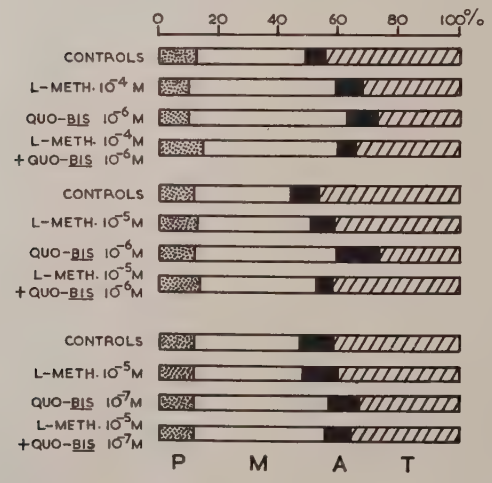

Fig. 2. Phase distribution after application of L-methionine (L-meth.) on quo-*bis*.

in which the mitotic inhibition is completely alleviated, the metaphase count fails to reach the control value. The accumulation of cells in metaphase is always accompanied in various degrees by cell oedema, pyknosis, fragmentation of chromosomes, occasionally by the appearance of tripolar cells and of binucleated cells in telophase, and by some effect on the resting cells.

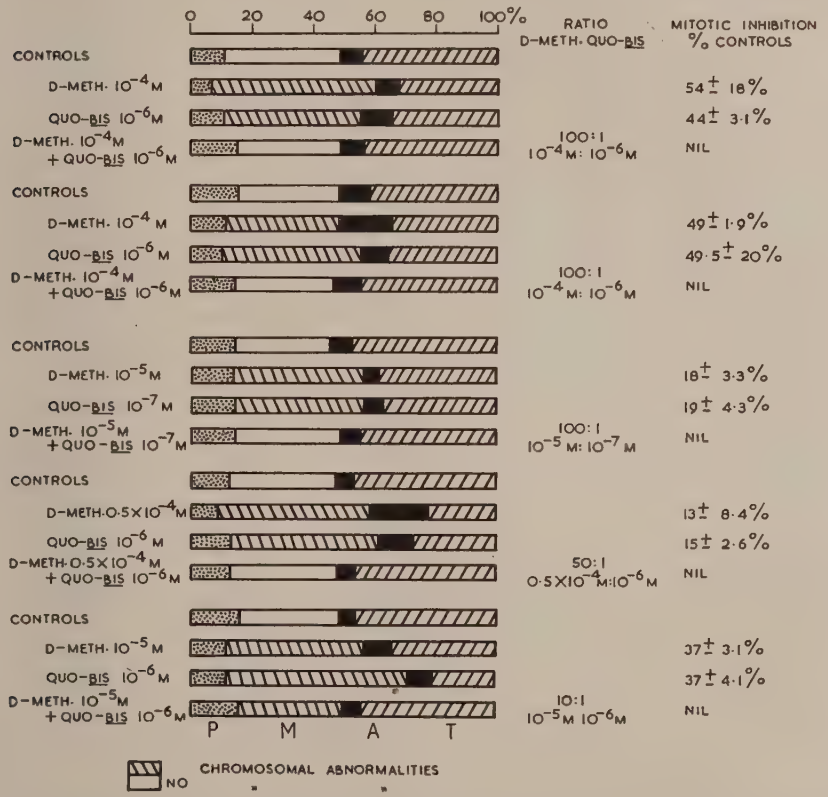

Fig. 3. Alleviation of the antimitotic effect of S:S(1:4-naphthoquinolyl-2:3)-*bis*-mercaptoacetic acid (quo-*bis*) by D-methionine (D-meth.).

D-*Methionine*.—Impressive results have been obtained by applying D-methionine to tissue cultures inhibited in their mitosis by quo-*bis*. This will be seen from Fig. 3.

In Fig. 3 five experiments are recorded. In three experiments the ratio

D-methionine / quo-*bis* is 100:1, the corresponding systems are D-methionine 10^{-4} *M* / quo-*bis* 10^{-6} *M* and D-methionine 10^{-5} *M* / quo-*bis* 10^{-7} *M*. In the fourth experiment the ratio D-methionine / quo-*bis* is 50:1, i.e. D-methionine 5×10^{-5} *M* / quo-*bis* 10^{-6} *M*. In the fifth experiment the ratio D-methionine / quo-*bis* is 10:1, i.e. D-methionine 10^{-5} *M* / quo-*bis* 10^{-6} *M*.

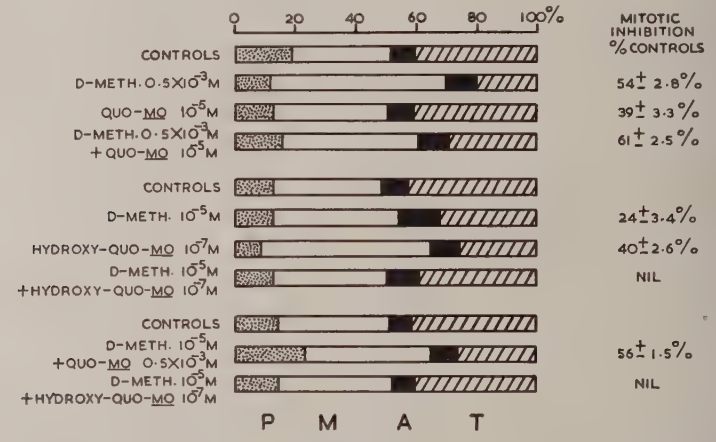

Fig. 4. Action of D-methionine on the antimitotic activity of quo-*mo* and hydroxy-quo-*mo*.

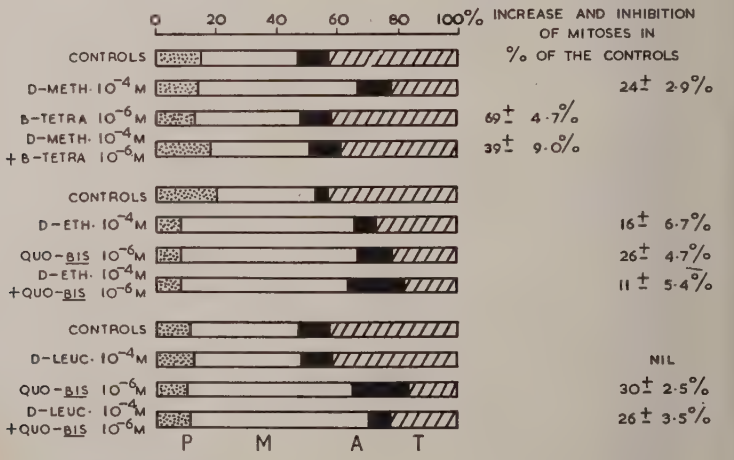

Fig. 5. Effect of D-methionine on the increase of mitosis caused by B-tetra and action of D-ethionine (D-eth.) and D-leucine (D-leuc.) on the mitotic inhibition caused by quo-*bis*.

In all five experiments the mitotic inhibition caused by quo-*bis* and by D-methionine separately is completely eliminated by the mixture of D-methionine / quo-*bis*. The same applies to the phase distribution down to the ratio 50:1. At the ratio D-methionine / quo-*bis* 10:1, however, cell oedema, enlarged cells in mitosis, clumped chromosomes and binucleated cells in telophase as well as a slight effect on resting cells are still encountered.

D-*Methionine applied to quo-mo, hydroxy-quo-mo and B-tetra.*—The action of D-methionine on the antimitotic activity of quo-*mo* and hydroxy-quo-*mo* is recorded in Fig. 4.

D-methionine exerts no effect on quo-*mo* as an inhibitor of mitosis but completely prevents the strong antimitotic action of hydroxy-quo-*mo*.

The increase of the mitotic count produced by B-*tetra* in tissue cultures is not changed by the addition of D-methionine (Fig. 5).

Amongst other D-amino-acids, only D-ethionine and D-leucine have been tested as alleviators of the antimitotic activity of quo-*bis* (D-ethionine in the system D-ethionine 10^{-4} *M* / quo-*bis* 10^{-6} *M*, and D-leucine in the system D-leucine 10^{-4} *M* / quo-*bis* 10^{-6} *M*). No alleviation has been observed with these substances (Fig. 5).

DISCUSSION

The experiments described in this paper show that the two groups of thioethers investigated—methionine on the one hand and hydroxy-quo-*mo* and quo-*bis* on the other—antagonise each other. Small amounts of the mercaptoacetic acid derivatives of 1:4-naphthoquinone alleviate the mitotic inhibition of L- and of D-methionine, and relatively large amounts of L- and D-methionine are necessary to cancel the mitotic inhibition of the mercaptoacetic adducts. As in other antagonistic processes (sulpha drugs and *p*-aminobenzoic acid [18], D- and L-leucine and D- and L-alanine) no stoichiometric proportions are followed in these reactions, the antagonistic ratio being 100:1 for L-methionine / quo-*bis* and for D-methionine / hydroxy-quo-*mo* and 10:1 for D-methionine / quo-*bis*.

The mitotic antagonism is manifested in three ways: (1) Partial alleviation of the mitotic inhibition, leaving arrest in metaphase and chromosomal aberrations more or less unimpaired (Table I and Fig. 2); (2) partial cancellation of the mitotic inhibition and of the arrest in metaphase, but no inhibition of the chromosomal aberrations (Fig. 1); and (3) removal of the mitotic inhibition, of the arrest in metaphase and of the chromosomal aberrations (total alleviation—Fig. 3). Fig. 3 shows further that the partial alleviation of

the second kind can be changed into total alleviation by raising the ratio D-methionine / quo-*bis* from 10:1 to 50:1.

The antagonistic activity of D-methionine is comparable to similar reactions of other D-amino acids. Thus D-leucine and D-valine exerted a growth inhibitory action on *L. arabinosus*, when the ratio D-amino acid to L-amino acid in the nutrient medium was greater than 40:1 [8]. D-Alanine inhibited the germination of *Bacillus anthracis* at the molecular ratio 1:10—inhibitor to promoter [12].

D-Methionine gives total alleviation of the antimitotic effect of hydroxy-quo-*mo* and of quo-*bis*, but no alleviation is observed with quo-*mo*. The two substances whose antimitotic actions are alleviated (quo-*bis* and hydroxy-quo-*mo*) are 2:3 substituted 1:4-naphtoquinone derivatives. Both are powerful mitotic inhibitors in concentrations of the order of 10^{-7} *M* whilst quo-*mo* shows antimitotic acitivity only at 10^{-5} *M*. The difference in alleviation indicates that the mechanism by which the *bis*-substituted 1:4-naphthoquinone adducts exert their antimitotic activity, is different from the mechanism by which quo-*mo* acts. As the lack of alleviation is complete, the difference is qualitative, not quantitative.

The total alleviation of the antimitotic effect of certain mercaptoacetic derivatives has only been observed so far with D-methionine, and D-ethionine and D-leucine are inactive. The question as to whether D-methionine is a specific alleviator of the antimitotic effects of these acids must remain open for the present.

In contrast to the total alleviation produced by D-methionine, L-methionine gives only partial alleviation even in a ratio L-methionine / quo-*bis* 100:1. But it may be remembered that the antagonistic effect of L-methionine can be increased by adding other metabolites: in *E. coli* the amount of *p*-aminobenzoic acid required to antagonise a given concentration of sulphamide is diminished threefold by methionine and another threefold on the further addition of xanthine and guanine [16].

The difference in the degree of physiological activity between D- and L-methionine is in agreement with the analogous behaviour of many other stereoisomers, although in the present experiments there is no evidence that D- and L-methionine react with the same receptor.

As far as L-methionine is concerned, one part of the reactions involved can be visualised, thanks to the important experiments of Cantoni and his collaborators [6]. According to these authors methionine forms an "active" compound which is known to be an energy-rich onium substance [7]. The constitution has been ascertained by synthesis [1, 2, 3] as adenosine–methio-

nine (AME). On the other hand the mercaptoacetic adducts of 1:4-naphthoquinone are easily oxidised in dilute solution to sulphoxides [9] which can be resolved into optically active compounds. Thus, without excluding other possibilities, it may be tentatively suggested that these two onium groups antagonise each other, the sulphoxides of the mercaptoacetic adducts acting as free sulphonium compounds or combined with other metabolites. The antagonistic chemical reactions between these two groups, have no stoichiometric relationship and appear to be displacement reactions in the sense suggested by Kuhn [13] and by Bersin [4].

The results of the present experiments seem to connect the antimitotic action of certain sulphydryl adducts of 1:4-naphthoquinone, via the alleviation of these effects by L-methionine, with "active methionine", i.e. adenosine-methionine, and thus with the methyl donating properties of L-methionine.

SUMMARY

1. The antimitotic effects produced in tissue cultures of chick fibroblasts by certain mercaptoacetic derivatives of 1:4-naphthoquinone (S-(2-hydroxy-1:4-naphthoquinolyl-3)mercaptoacetic acid, called hydroxy-quo-*mo*, and S:S(1:4-naphthoquinolyl-2:3)-*bis*-mercaptoacetic acid, called quo-*bis*) and by D-, L- and DL-methionine, is alleviated by applying a mixture of the mercaptoacetic adducts and the methionines to the growing cells.

2. Total alleviation is obtained in the system D-methionine / quo-*bis* at a ratio D-methionine / quo-*bis* greater than 10:1. The system D-methionine / hydroxy-quo-*mo* gives complete alleviation at the ratio D-methionine / hydroxy-quo-*mo* 100:1.

3. With L-methionine / quo-*bis*, only partial alleviation is achieved even at a ratio L-methionine / quo-*bis* 100:1. The mitotic inhibition is completely cancelled, but arrest in metaphase is only partially decreased and there is no inhibition of the chromosomal abnormalities.

4. In the system D-methionine / quo-*mo* no alleviation has been found.

5. D-Ethionine and D-leucine fail to antagonise the antimitotic effect of quo-*bis*.

6. The increase of the mitotic count observed after application of the tetra-mercaptoacetic derivative of 1:4-benzoquinone remains unimpaired by D-methionine.

7. It has been pointed out that the alleviation of the antimitotic action of certain sulphydryl adducts of 1:4-naphthoquinone by means of L-methionine,

establishes a connection between the antimitotic effects of these compounds and "active methionine" (adenosine–methionine) and thus with the methyl donating properties of L-methionine.

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THE SIGNIFICANCE OF POLYPLOIDY FOR THE EVOLUTION OF MOUSE TUMORS

STRAINS OF THE TA3 MAMMARY ADENOCARCINOMA WITH DIFFERENT PLOIDY

A. LEVAN

Cancer Chromosome Laboratory, Institute of Genetics, Lund, Sweden

Received June 12, 1956

It has long been known that tumors differ from normal somatic tissue by a general tendency to mitotic instability. Only after it was found that the ascitic forms of many tumors were especially favorable for the close study of the chromosomes, has it become possible to express these more or less vague observations in a more exact manner and to define the differences existing in terms of modern cyto-genetics.

Thus, the study of ascites tumors in rats and mice revealed that the tumors consist of a cell population of genetically heterogeneous elements. This heterogeneity is directly observable as a chromosome number variation around a modal number, the so-called stemline number. In addition, there is also a smaller fraction of cells with a similar variation around the double stemline number. These types of variation are formed by chromosomal mechanisms acting in the tumor population, such as non-disjunction, endomitosis, *c*-mitosis, spindle multipolarity, etc.

While some authors claim that a high degree of numerical chromosome variation also occurs in normal somatic tissues, most of the available evidence, including our own experience from regenerating rat and mouse liver and from human embryonic lung fibroblast cultures [14, 15], indicates a high degree of mitotic constancy in somatic meristems. The conditions may be different in disintegrating or specialized tissues which no longer need go through normal mitosis for their permanence: while mechanisms for chromosome doublings act in normal tissues, non-disjunction is probably of little importance here.

In tumors the stemline number generally deviates from the normal somatic number of the host species. In mouse tumors the stemline number is usually higher than $2n$, the highest measure of viability in mouse tumors apparently being realized at and below the tetraploid number. In adapting itself to the

environment the stemline makes use of the genetic heterogeneity of the cancer cell population and gradually shifts over to an optimal number.

The mitotic instability of tumors includes chromosome breaks and new combinations of broken ends, i.e. structural variation. This kind of heterogeneity is also a contributory factor in the evolution of the tumors in that structurally "new" chromosomes enter the stemline. Many tumors are characterized by such distinctive chromosomes, for instance the *A*, *B*, min_A, min_B of the hyperdiploid Ehrlich, the *V* chromosomes and the minutes of certain strains of the tetraploid Ehrlich and Krebs 2, the large *V* chromosomes of the Yoshida rat sarcoma. No similar structural variation has been observed in normal somatic tissue, unless we include the very rare cases of self-perpetuating ring chromosomes [e.g. 11, 1]. The analyses performed by Tjio and the present author in regenerating liver have produced no evidence of structural variation.

It is comparatively easy to understand the extent of the numerical variation within a tumor cell population and its significance for the development of the tumor. On the other hand, the role of structural rearrangements is more difficult to determine. Judging only from new chromosomes in the tumors, which can be identified immediately, structural changes may be considered to be of comparatively little significance.

A first approach to this problem was made two years ago by Hauschka and myself [4] on some twenty different strains, stock tumors and clones, of the near-tetraploid Ehrlich and Krebs 2 mouse tumors. In a number of metaphase plates all chromosomes were measured and the distribution of the chromosome lengths was compared with a representative material of spermatogonial chromosomes from mouse. From this procedure we got an idea about the structural changes during the development of the tumors.

Much to our surprise the distribution of chromosome lengths of the tumors deviated in a striking and consistent way from that of the spermatogonial cells: every tumor idiogram measured had a decidedly wider length distribution than had the spermatogonial cells, and both smaller and longer chromosomes were present in the tumors, while the classes with intermediate lengths were under-represented. Since it may be inferred that each observable structural change corresponds to many changes that cannot be observed with the crude method used, our results suggested that the tumors studied had undergone far more profound structural remodelling than indicated only by the immediately detectable distinctive chromosomes. Therefore, besides the gross structural variation directly observed, another less striking variation is acting which may be called the crypto-structural variation.

All tumors in which the crypto-variation was discovered were near-tetraploid. We were thus unable to decide whether the ability of accumulating cryptostructural changes was characteristic of tumor tissue versus normal tissue, or whether it was a property of tetraploidy versus diploidy. Certain facts favored the latter alternative. Thus, already in an earlier investigation [10] we noticed that deviating chromosome types occurred more frequently in near-tetraploid than in near-diploid tumors. A definite answer to the question is given only when comparing diploid and tetraploid idiograms of normal mouse tissue on one side, and diploid and tetraploid forms of the same tumor on the other side.

In the present paper the latter comparison has been made for one tumor, viz. the TA3 mammary adenocarcinoma, which exists in near-diploid and hypotetraploid ascitic forms. The purpose of the present investigation is to compare the chromosome morphology of these two types with the normal mouse idiogram in order to determine whether morphologically detectable differences in chromosome structure occur between the germline chromosomes of the mouse and the tumor strains, and, in case there are differences between the tumor strains, which of them exhibits the greatest deviation from the mouse idiogram. It is necessary first briefly to mention some data on the origin of the different tumor strains and on the numerical conditions of the strains.

MATERIAL

The mammary adenocarcinoma of TA3 originated spontaneously in 1949 in an A/Ha female mouse at the Institute for Cancer Research in Philadelphia. It was propagated subcutaneously as a solid growth in A mice. It was transformed into an ascites from Hauschka's transfer on two separate occasions, viz. from transfer generation 16 [7], and from transfer generation 34 [2]. The former ascites strain was brought to Stockholm in 1951 and has since then remained there, while the latter strain has remained in Philadelphia and Buffalo.

Klein [8] later found that the former strain, here called TA3Kl, which was supposedly diploid at the start, had a nuclear volume and a DNA content which were more compatible with a tetraploid than with a diploid condition. The chromosome counts for this strain, recorded below, were made in 1954 on generation 187. They verified the conclusion drawn by Klein from his DNA determination.

One sample of the TA3Kl was sent in 1952 to the Lederle Laboratories, Pearl River, N.Y., where the strain was continued in serial transfer by Dr. H. Koprowsky. This strain, here called TA3KlKo, was also brought to Philadelphia, where Dr. Hauschka [2] determined it as hypotetraploid. My fixations of this strain in 1954 confirmed his result (see below).

The strain from the second ascites transformation, here called TA3Ha, was studied chromosomally in 1953 in generation 50 [10]. The tumor had a diploid mode; among

128 cells 109 had numbers close to $2x$, one cell had 54 chromosomes, and 18 cells had near-tetraploid numbers. The *s* mode had the following constitution:

$2n$	38	39	40	41	42	
Number of cells	1	1	23	15	3	Mean 40.4

Thus, some tendency to hyperdiploidy appeared already at that time.

During my stay in Philadelphia in 1954 the TA3Ha was still near-diploid. It was then somewhat less strain-specific and grew progressively in a certain percentage of the animals of seven foreign host strains [2]. The material for the chromosome analyses below comes from generation 37 in Swiss mice. At that time the tumor in home strain was in generation 170–200.

The host-specificity of TA3Kl is still high; it grows progressively only in home strain [8]. According to Hauschka TA3KlKo is less specific than TA3Ha. At trials with the same seven host strains as tested against TA3Ha, TA3KlKo grew in all of them and killed 100 per cent of the animals in two of them.

Chromosome Numbers

Exact chromosome counts were made in 100 cells of each TA3Ha and TA3KlKo, and in 200 cells of TA3Kl. The results are recorded in Table I.

All three strains have clearly departed from the somatic mouse number 40. TA3Ha is still close to it, while the other two have settled in the region halfway between $3x$ and $4x$. The distribution of chromosome numbers is much narrower in TA3Ha, 79 per cent of the cells having 41 chromosomes. This strain undoubtedly has another chromosome number distribution than in 1952, although the change in the distributional mean is insignificant (from 40.4 to 40.9). Hauschka [2] reports that this strain had, around generation 120, a stronger tendency to hyperdiploidy than indicated by the present counts, in fact 94 per cent of the cells were hyperdiploid. This indicates that small

Table I

Percentages of cells with the following chromosome numbers within the *s* region in the three TA3 strains analyzed.

Strain	Chromosome number																			Average number	Number of cells counted
	40	41	42	43	64	65	66	67	68	69	70	71	72	73	74	75					
TA3Ha	13	79	7	1	—	—	—	—	—	—	—	—	—	—	—	—				40.9	100
TA3Kl	—	—	—	—	1	3	10.5	17	36	18.5	8	3	2.5	—	0.5	—				68.1	200
TA3KlKo	—	—	—	—	—	—	1	1	4	17	36	19	9	8	4	1				70.5	100

fluctuations to and from the modal number take place. One count is never sufficient to characterize a tumor.

The other two strains are numerically quite distinct from TA3Ha. Although they are hardly different statistically, they are no doubt different biologically. This is particularly clear when their chromosome morphology is taken into account. The TA3Kl and the TA3KlKo have drifted apart subsequent to their separation in 1952. They do not only differ functionally, as in their host requirements, but also morphologically, in chromosome number distributions and chromosome structure. It is of interest, however, that both of them still maintain the same nucleolar type characteristic of the original solid TA3 tumor ([7], Figs. 18–21). This condition is characteristic enough to dispose of any doubts as to the identical origin of the three TA3 strains. The mode of growth in small colonies, characteristic of the original TA3 ascites tumors, has been retained in TA3Ha and TA3Kl but has been practically lost in TA3KlKo [3].

Chromosome Structure

As is well known, the mouse germline karyotype consists of exclusively telocentric chromosomes (Fig. 1 *a*). This makes the mouse chromosome complement suitable for quantitative treatment. In fact it is comparable to a metacentric chromosome complement of only 20 chromosomes. The chromosomes vary in length gradually from about 2 μ to 5 μ, with most chromosomes in the intermediate classes. Their lengths consequently fall in a distribution similar to the random distribution around a mean at 3.5 μ. This suggests that it may not be entirely out of place to visualize an optimal length of the mouse chromosome, and that a chromosome length deviating so much to the left and so much to the right of this optimal length is tolerated in the karyotype, while more extreme deviations will lead to chromosome mechanic trouble at mitosis or meiosis.

An instance of the TA3Ha idiogram is pictured in Fig. 1 *b*. It shows close agreement with the mouse karyotype. It contains, however, two distinctive chromosomes, represented at the end of the row of Fig. 1 *b*. These are small, usually not the very smallest ones as in Fig. 1 *b*, but mostly in the range of nos. 3 and 4 from the end of the idiogram. In Fig. 1 *e–h* they are drawn together with the largest and smallest chromosome of four cells and in Fig. 1 *i* they are represented in eight different cells. They were noticed already in our earlier analysis of this tumor ([10], Fig. 1 *G, H*) and are quite stable members of the stemline complement. Although their appearance indicates a subterminal location of the centromere, the constriction is more probably

a secondary constriction. At anaphase these chromosomes proceed to the poles in a straight position—not bent at the constriction as would have been the case if the constriction had held the centromere. Probably they correspond to one pair of mouse chromosomes having a proximal differential segment,

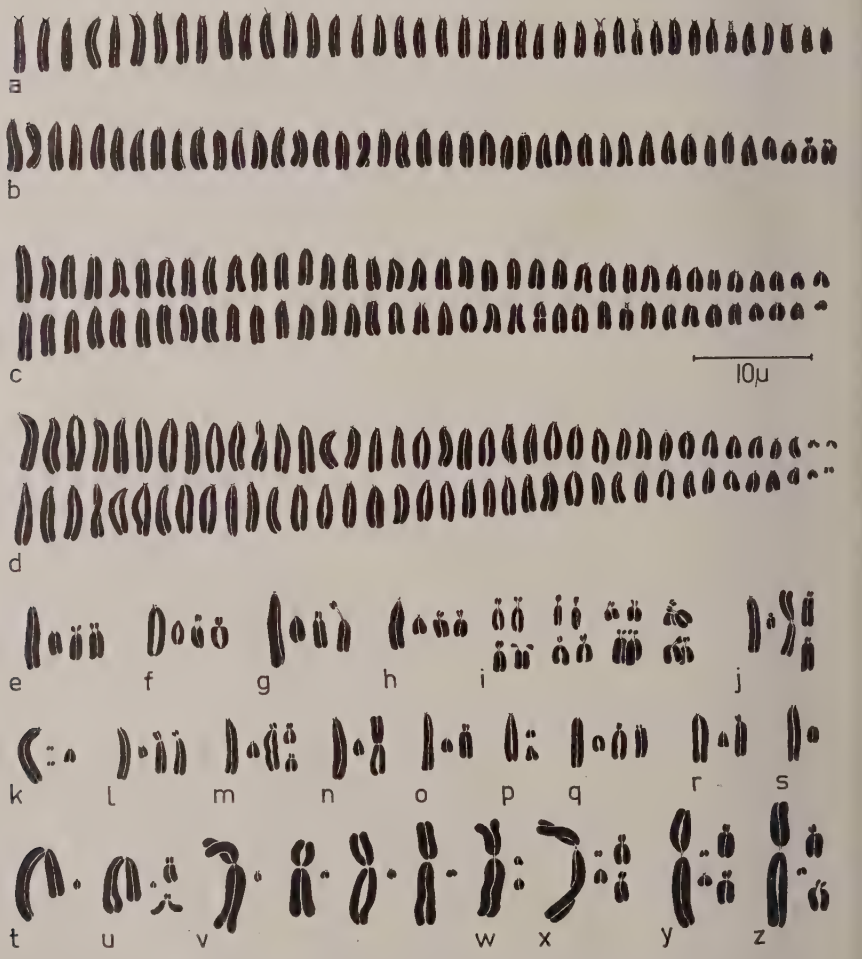


Fig. 1. *a*, Typical idiogram of the spermatogonial chromosomes of the mouse; *b*, idiogram of the hyperdiploid TA3Ha; *c*, the hypotetraploid TA3KlKo; *d*, TA3Kl. Landmark chromosomes of *e–i:* TA3Ha; *j–s:* TA3KlKo; *t–z:* TA3Kl. ×1750.

a number of which are found in particular among the smaller mouse chromosomes. Four of them are seen in Fig. 1 *a*. Under certain conditions all mouse chromosomes may exhibit such proximal segments, for instance after chemical treatments ([13], Fig. 14 *b*), and they are probably manifestations of the centric heterochromatin. It may be concluded that except for the deviating chromosome number no morphological differences are apparent between the chromosomes of the mouse and those of the TA3Ha strain.

Typical idiograms of the two hypotetraploid strains TA3Kl and TA3KlKo are given in Fig. 1 *d* and *c*, which show that the extremes in chromosome length of the mouse idiogram have been exceeded. This is seen most clearly in the region of the small chromosomes, but it is found also at the other end of the idiogram. Several cases of size extremes from different cells are represented for TA3Kl in Fig. 1 *t–z* and for TA3KlKo in Fig. 1 *j–s*. These pictures illustrate the far higher variability of chromosomes of hypotetraploid strains as compared with the hyperdiploid TA3. The smallest chromosome present is always much smaller than that of the spermatogonial cells or of TA3Ha, but it varies from 0.4 to 1.5 μ, the longest chromosome varying from 4.5 to 6.7 μ in the measured idiograms. The tolerance limits for chromosome length set in the normal mouse idiogram have obviously been considerably widened in the hypotetraploid strains.

Other distinctive chromosomes of TA3Kl and TA3KlKo have been included in the chromosome samples drawn in Fig. 1 *j–z*. Chromosomes with proximal differentiations, recalling the two distinctive chromosomes of TA3Ha, are often present in both strains. In some cases their morphology suggests that they may be identical with them (as in Fig. 1 *m, u, x–z*), but in other cases they are clearly different (Fig. 1 *j, l, r*). Although they do not occur with the same regularity in the hypotetraploid strains as in TA3Ha, it is possible that they represent in all three strains descendants of a distinctive chromosome present already in the original solid TA3. They were far more frequent in TA3KlKo than in TA3Kl.

Metacentric chromosomes were observed now and then; in TA3KlKo they were found only occasionally (Fig. 1 *j, n*), but in TA3Kl the largest chromosome arm of the entire complement was very often joined to an intermediate-sized arm into a very striking metacentric formation. This was not the case in the idiogram of Fig. 1 *d*, but many instances are given in Fig. 1 *t–z*. It should be noted that TA3Kl represents a somewhat more extreme deviation from the normal mouse chromosomes than TA3KlKo.

In order to make a more accurate comparison between the karyotypes here analyzed, measurements were made of all chromosomes of five meta-

TABLE II

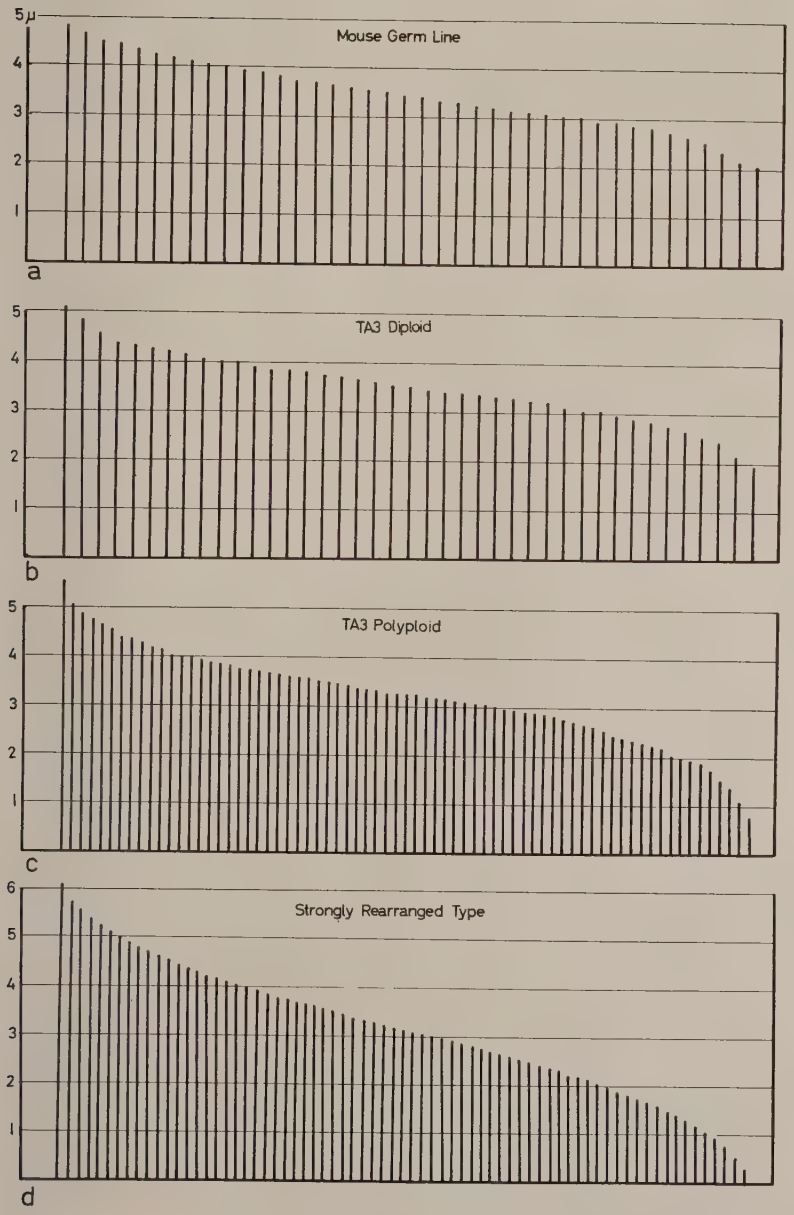
Chromosome length (in 0.1 μ) in ten spermatogonial metaphases of the mouse and in seventeen metaphases of the TA3 strains.

Material	Chromosome	Metaphase no. 1	2	3	4	5	6	7	8	9	10	Average
Spermatogonial cells	Smallest	18	19	17	19	20	21	20	21	22	24	20
	Largest	46	43	46	45	50	47	48	52	53	53	48
	Average	31	32	33	34	34	35	36	37	38	38	35
TA3Ha	Smallest	17	19	19	20	23	—	—	—	—	—	20
	Largest	43	46	46	55	65	—	—	—	—	—	51
	Average	29	31	33	39	43	—	—	—	—	—	35
TA3Kl	Smallest	5	6	5	7	4	7	8	—	—	—	6
	Largest	55	55	59	59	64	54	67	—	—	—	59
	Average	31	32	32	33	34	34	37	—	—	—	33
TA3KlKo	Smallest	8	14	7	14	11	—	—	—	—	—	11
	Largest	50	45	46	54	53	—	—	—	—	—	50
	Average	27	29	31	31	34	—	—	—	—	—	30

phase cells of each of TA3Ha and TA3KlKo, and of seven cells of TA3Kl. The values obtained were compared with similar measurements of ten spermatogonial metaphases made in Philadelphia in connection with the analysis of the Ehrlich and Krebs 2 material referred to above [4]. In Table II the extremes and averages of each series are recorded. It is evident from this table that the hyperdiploid TA3Ha shows close agreement with the spermatogonial chromosomes, while the hypotetraploid strains have greater extremes. There was some indication that the hypotetraploids had a somewhat shorter average chromosome length than the spermatogonial cells and the hyperdiploid tumor, but with the small material so far available no definite conclusion has been reached on this point.

The entire material of chromosome measurements was used for constructing average idiograms of the different karyotypes (Fig. 2). Within the three groups—(1) spermatogonial cells, (2) hyperdiploid strain, and (3) the two hypotetraploid strains added together—the chromosome lengths of each cell

Fig. 2. Average idiograms of *a*, ten instances of the spermatogonial metaphases of the mouse; *b*, five instances of the TA3Ha tumor; *c*, twelve instances of the TA3Kl plus TA3KlKo; *d*, a hypothetic idiogram representing the end result after random structural interchange between mouse germline chromosomes of average size.


5μ
Mouse Germ Line
4
3
2
1
a
5
TA3 Diploid
4
3
2
1
b
5
TA3 Polyploid
4
3
2
1
c
6
Strongly Rearranged Type
5
4
3
2
1
d

were listed from the longest to the shortest. In the hypotetraploid group (3) different chromosome numbers present were adjusted by the omission or duplication of one or more average chromosomes. In the case of the spermatogonial chromosomes it would have been preferable to give one representation for each of the 20 pairs present, but since the identity of the sex heteropair is not known, this was impracticable. The material available added up to ten series of 40 mice chromosomes, five series of 41 TA3Ha chromosomes, and 12 series of 71 TA3Kl plus TA3KlKo chromosomes, in all 1457 measurements. In each series the chromosomes were numbered after falling length. Averages were calculated for each group starting with chromosome no. 1 and going down to the last chromosome of the group. This procedure, though undoubtedly suffering from gross inexactitude, is as close as one can come at present in obtaining average lengths of homologous chromosomes of different cells. It will give a somewhat better representation of the true situation than the mere inspection of individual plates.

Fig. 2 gives a clear idea of the conditions: While the idiograms of the two diploid materials, the spermatogonial cells and the TA3Ha, practically coincide, the idiogram of the hypotetraploid strains is strikingly different. If all three idiograms had been adjusted to the same average chromosome length, it would have been evident that the hypotetraploid idiogram coincides with the others in the middle region, but differs at both ends. Since the average length of the hypotetraploids was somewhat lower than that of the diploids the differences of the idiogram are exaggerated for the right end and diminished for the left end. At any rate, the situation is made perfectly clear by the average idiograms: the near-diploid strain is still largely concordant in chromosome morphology with the ordinary mouse chromosomes, while the hypotetraploid strains have departed from this condition. This departure involves the acquirement of an essentially different karyotype. This is morphologically undistinguishable from the type found in the near-tetraploid strains of Ehrlich and Krebs 2 tumors, and represents a general karyotype probably attained in time by any near-tetraploid mouse tumors.

One may speculate upon the nature of the structural rearrangements underlying the differences observed between the two types of idiogram, here called for brevity the diploid and the tetraploid idiogram, respectively. As is well known, each interchange between two chromosomes may give rise to one pair of extreme chromosome types, if the points of interchange are located in different regions of the two chromosomes. Biarmed chromosomes should result very rarely, after inverted interchange with the points of breakage in both chromosomes within the centromere. All other inverted inter-

changes would give rise to one dicentric and one acentric chromosome. Since there seems to be no reason why highly asymmetric changes should occur more frequently than more symmetric ones, the observation of some morphologically extreme chromosome types may indicate that a great many rearrangements have taken place.

On a suggestion of Dr. Östergren an attempt was made to investigate in what direction an idiogram of equal-sized chromosomes changes when exposed to numerous structural interchanges. Two chromosomes of the same length were divided into ten regions each, and random interchanges were supposed to take place between them with the ten regions as units. This resulted in the idiogram of Fig. 2*d*. Thus, random interchanges in ten regions per chromosome will change an idiogram of equal chromosomes into an idiogram with chromosome lengths varying between 1.9 and 0.1 of the original chromosome length. It is evident that this theoretical idiogram represents a final stage in the same series of changes, in which the polyploid idiogram of Fig. 2*c* is an earlier step and in which both the normal mouse chromosomes and the diploid tumor chromosomes represent still earlier steps. This supports the conclusion, drawn from other considerations, that polyploid tumors undergo heavy structural rearrangements during their development. The diploid tumor idiogram (Fig. 2*b*) shows a very insignificant change in the same direction.

The development briefly outlined here is, of course, an approximation. The changes must, for instance, be supposed to occur continuously, so that any new chromosome type formed is immediately exposed to new rearrangements. The differences between the extreme chromosome lengths should therefore be expected to grow gradually. Evidently an equilibrium is established between the tendency to rearrangements and the lowered viability of cells with extreme chromosome types. Chromosomes exceeding a certain length will cause mechanical trouble.

Actually the tetraploid idiogram observed may be constructed by allowing for an increased number of interchanges in intermediate chromosome regions instead of complete randomness. On the assumption of the following number of interchanges: none in regions 1 and 10, one in regions 2, 3, 8, and 9, and two in regions 4, 5, 6, and 7, an almost exact duplicate of the idiogram of Fig. 2*c* is produced.

If the mutual position of the two interchanging chromosomes is not at random a similar favoring of the intermediate-sized chromosomes results at the expense of the extreme sizes. If there is a tendency to juxtaposition of the chromosomes with the centromeres on the same level, interchanges in

all regions will result in new chromosomes identical in size with the old ones. The gathering of the chromosomes into telophase nuclei favors such a juxtaposition, which may very well be maintained during resting stage.

Cyto-genetic Outlook

The present investigation is an instance of a problem within cyto-genetics of tissues, which is very common in the cyto-genetics of organisms, namely, the comparative analysis of related strains with different chromosome numbers. Even though in the present case the unit of the population is the individual cell instead of the individual organism, the present problem belongs within the realms of polyploidy research; more specifically it is a problem of evolutionary changes following the induction of autopolyploidy.

One tumor strain has maintained a near-diploid stemline, while another strain, derived from the same solid tumor, has acquired a hypotetraploid stemline. We cannot reconstruct the detailed course of events leading to the hypotetraploidy of this latter strain. It may have started from a chromosome-doubled cell within the diploid population, and the tetraploid stemline thus established may gradually have lost a number of chromosomes, while adapting itself to its new condition. We know that such "diminutions" of the chromosome number may occur with tetraploid tumors; the Krebs 2 and Ehrlich stocks, for instance, lost several of their 80 chromosomes after their transfer from Stockholm to Philadelphia [4, 9].

One main difference in the chromosome variation between populations of tumor cells and of organisms is the much greater tolerance to abnormal chromosome complements of the former. It appears as if the significance of many individual chromosomes were more or less negligible in tumors. However, the vital importance of certain chromosomes to the tumors is shown by the fact that various freaks, such as very small minutes, ring chromosomes etc., which may seem to be a handicap for the smooth mitotic function of the cells carrying them, are nevertheless maintained for long periods in tumor stemlines. In any case, it is a general characteristic of tumors that the modal stemline continuously produces cells with deviating chromosome numbers, and that these cells are able to survive one or more mitotic cycles. There is even reason to believe that more or less permanent secondary lineages with deviating chromosome numbers may exist alongside the modal stemline in a tumor. In other words, the stemline may be heterogeneous, consisting of a number of viable lines growing side by side. Each of them is adapted for an environment including all the others. The result of this mutual

co-adaptation is that individual types of these lines, when isolated, may exhibit quite new properties. Thus it may happen that a certain clone grows more vigorously, and kills the host in shorter time than the mother strain [2].

This careless variation in chromosome number characteristic of tumors is banned in the germ-track and probably in the normal tissues of organisms. It is true that monosomics, polysomics, and polyploids occur in higher plants, but the immediate effect of the loss or addition of individual chromosomes is lowered viability or lethality for the cells concerned, and sterility for the individuals. In higher plants the addition of complete basic chromosome sets means a much lesser disturbance of the genetic balance than the addition of single chromosomes. Chromosome-doubled types are usually viable and capable of sexual reproduction, while mono- or polysomatic types, at least on the diploid level, are always highly sterile, if viable at all. New polyploids, however, have to pass a thorough genic and chromosome structural remodelling in order to attain selective superiority, or even ability of surviving; that is a conclusion that can be drawn from the experiences of polyploidy breeding. When more than one basic chromosome number is present among related forms, this condition has arisen through long periods of evolutionary changes, and certainly involves much more complex changes than the mere loss or gain of one or more unchanged chromosomes.

It seems that in higher plants autopolyploid genotypes in some respects have inherent a higher degree of evolutionary potentialites than corresponding diploids. Mutations in a certain direction may accumulate more freely in the polyploids, which means that they are adaptable to more extreme conditions. This is so because each change in a chromosome of an autotetraploid is buffered by the presence in the genotype of three unchanged chromosomes of the same kind. This may be the background to the fact that the proportion of polyploids to diploids is higher in regions of the world with extreme conditions, as in the arctics, deserts, etc.

The instability of the mitotic mechanism in tumors with the consequent chromosome variation undoubtedly constitutes a key phenomenon to the progression to which tumors are normally subjected. The conservative influence on the karyotype of sexual mechanisms is, of course, absent in the tumors. In addition, the cancer cells lead a very specific type of parasitic life, inside a host built up of cells of a descent common with their own, surrounded by a continuous supply of everything needed for their growth and propagation. Under these conditions natural selection will favor those genotypes that have improved their autonomy relative to the host control, that is those able to grow, multiply and invade new territories. On the other

hand, many other qualities and functions of the cells, governed by the chromosomes, are no longer of importance to the tumor. This may be the background both to the gradual dedifferentiation of the tumor cells and to the ease with which some chromosomes are lost or duplicated, while other chromosomes, or even very small centric fragments, seem indispensable. In this connection reference is made to Schultz [12], who, introducing the concept of genic "adaptation", thinks it possible that the majority of genes in old specialized tissues may be in "a state of generalized function concerned with gene reproduction and the mitotic process". This should correspond cytologically to "the assumption of the properties of heterochromatin by the regions of chromosome involved".

The common chromosome number variation around the modal stemline number, giving rise to cells differing in one or a few chromosomes from the stemline, is comparable to the formation of mono- and polysomics in higher organisms. It is an ephemeral chromosome number variation in both cases.

The permanent shifting over of the stemline to a new modal number may be compared with the development of a new basic number in an organism. As stated above, this is a very complex process in organisms. In tumor cells it means that a new stemline is formed, superior to the old one in rate of growth and division. This cannot be achieved by a mere gain or loss of whole unchanged chromosomes: additional intra- and interchromosomal changes in many chromosomes of the whole complement are necessary. The actual presence at all times in the population of cells with deviating chromosome numbers enhances the gradual production of cell lineages, capable of competition with the actual stemline. Gradually one of these sidelines may become equal in viability to the stemline, and a small change in the environment is all that is needed to shift the balance over in favor of the new chromosome number. The presence in the population of many viable and well-balanced, but different, sidelines is in its effect comparable to a very efficient defence mechanism of the tumor against changes in the environment.

The genetic heterogeneity of the cancer cell population together with the genetic variation assiduously going on at all times makes the tumors very responsive to the environment. Any environmental change effects a selective pressure on the population favoring cell lines capable of growth, or at least of survival, under new conditions. These lines immediately start adjusting themselves genetically to an ever better command of the new situation. Gradual adaptations, taking place step by step indicate polygenic changes including all kinds of changes from point mutations to gross structural re-

arrangements, and even heritable changes in plasmagenes. On the tetraploid level certainly the construction of the karyotypes is entirely compatible with a history involving an immense number of structural rearrangements.

The acquired ability of growth as ascites, for instance, is often realized as a stepwise adaptation. As demonstrated by George and Eva Klein [7, 5 and elsewhere] the difference between typical S cells (those capable of living only in solid form) and A cells (those preferring life as ascites) is very complex and of a genetic nature. There are many steps between the two conditions: capacity of provoking exudate formation, to survive in the exudate, to grow and divide, and finally to prefer life in the exudate. The whole picture suggests a polygenic difference between S and A. This conclusion is strengthened by the fact that, except for some neoplasms of the hemo- and lymphopoietic system, it is the rule that mouse ascites tumors are polyploid. In one case, the S3A tumor [6], the spontaneous conversion into ascites was known to have taken place concomitantly with an increase in chromosome number from diploidy to hypotetraploidy.

The ease with which new stemlines are established, differing from the previous one with one or a few chromosomes, detracts from the significance of eupolyploidy as an evolutionary factor in tumors. Even if one of the main avenues of evolution in tumors, as in higher plants, is chromosome doubling, both because of the frequent occurrence in the population of $2s$ cells and of the superior evolutionary potential of these cells, tetraploidy in mouse tumors is often only a transient stage on the way to hypotetraploidy. Secondary numerical variation is often superimposed on the chromosome doubling.

Many of the current genetic terms are somewhat awkward when adapted to cancer cell populations, mainly because of their genetic instability. Thus, the cell lineages originating from one single cell, isolated from a population, are called clones, although very soon after their establishment they will form populations of a heterogeneity comparable to that of the original population. Strictly speaking, all cells of tumors derived from the same individual animal are clone cells by definition. Likewise it seems somewhat inappropriate to speak of the polyploid tumor strains as autopolyploids, even though we know that genealogically they are autopolyploids. The present observations on the considerable structural differences between the hypotetraploid karyotype and the near-diploid karyotype stress this point. On the other hand it is advisable for practical reasons to use terms such as triploid and tetraploid for stemlines having the $3x$ and $4x$ chromosome numbers, without implying that they should have the exact structure of three or four basic sets of chromosomes. I have used here the term hypotetraploid for the strains with modes

at 68–71 with the understanding that they may well be hypodiploid for some chromosomes or chromosome segments and hypertetraploid or more for others.

The most significant observation of the present study concerns the demonstration that the hypotetraploid strains of the TA3 tumor have a karyotype essentially different from that of the mouse, while the near-diploid strain still largely maintains the mouse karyotype. The hypertriploid karyotypes have undergone similar changes as observed earlier by Hauschka and myself in two near-tetraploid mouse tumors, and it is likely that such aberrant karyotypes will gradually be adopted by any mouse tumor settling at the tetraploid level.

As has been discussed in an earlier paper [9], the increased degree of genotypic diversity of polyploid tumors as compared with diploid ones is in good agreement with conditions in higher plants that were briefly mentioned above. The evolutionary significance of tetraploidy in tumors is that it endows the stemline with the capacity of accumulating in the population a much more diverse supply of genotypes than is possible on the diploid level. This diversity forms the basis for further evolutionary developments. The acquirement of a polyploid stemline is therefore an important milestone for the tumors on their way towards progression.

SUMMARY

Chromosome analyses were made in one hyperdiploid and two hypotetraploid strains of the mammary adenocarcinoma ascites TA3 of the mouse. The karyotypes of these three strains were compared with that of the mouse germline. It appears that, while the karyotype of the near-diploid strain agrees closely with the mouse karyotype, the two hypotetraploid strains have karyotypes highly deviating from the mouse karyotype, suggesting that structural rearrangements are accumulated more freely on the polyploid level than on the diploid level. The significance of this for the understanding of some evolutionary trends in tumors as compared with organisms is discussed.

This investigation was supported by grants from the U.S. Public Health Service and from the Swedish Cancer Society, for which I express my deep gratitude. Two of the employed tumor strains were fixed in Philadelphia at the laboratory of Dr. T. S. Hauschka, and the third strain was fixed in Stockholm at the laboratory of Dr. G. Klein. My sincere thanks are due to Drs. Hauschka and Klein for the material and for their constant interest and helpfulness.

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CELL DIVISION WITHOUT MITOTIC APPARATUS IN SEA URCHIN EGGS

Y. HIRAMOTO

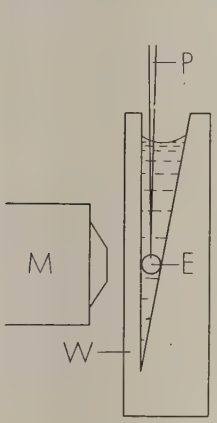
Misaki Marine Biological Station, Miura-shi, Kanagawa-ken, Japan

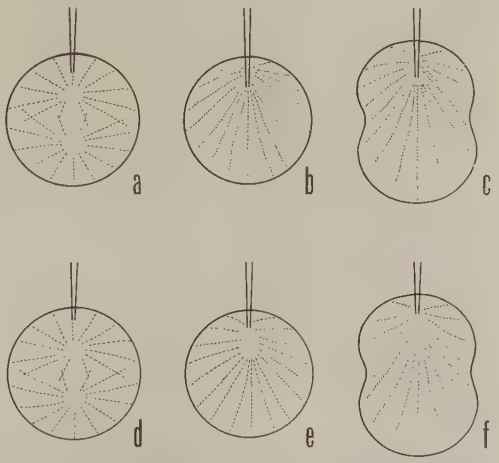
Received June 15, 1956

For a long time, the mechanism of cell division has been an interesting problem for biologists and many theories have been put forward by various investigators. From the viewpoint of the location of the motive force of division, these theories can be roughly classified in two groups: cortical activity theory and endoplasmic activity theory. According to the cortical activity theory, the motive force of cell division is located in the cortical region of the cell. The surface tension theory [10], the contracting ring theory [1, 5, 6, 8], and the expanding membrane theory [9] are included in the former category. According to the endoplasmic activity theory, the cell surface is pulled passively into the furrow by an active function of the internal structures such as the spindle and the asters. The traction fiber theory [4], the astral gelation theory [3], and the radiate aster theory [2] are included in the latter category. Recently, experiments favourable for the cortical activity theory have been reported in amphibian and in echinoderm eggs [11, 12]. In amphibian eggs, the furrow can extend and deepen when separated from the interior of the cell by a strip of cellophane, thus little or no part can be played by the mechanical pulls of astral rays attached to the cortex [12]. According to Swann and Mitchison [11], cleavage of sea urchin eggs can take place when the asters and spindle have been completely abolished by the treatment with concentrated colchicine during the mid-anaphase. The motive force of division can clearly not be derived from the spindle or asters if these structures have *completely* disappeared. Although Swann and Mitchison judged the disappearance of the spindle and asters as a result of observation with the polarization microscope and viscosity measurement, some doubts are left on the possibilities that some parts of these structures may still remain and that some specific effects of colchicine may be involved. Therefore, it is desirable to test by more direct methods whether or not the cell can divide without the spindle and the asters. The author attempted this and observed that cleavage did take place in sea urchin eggs from which the spindle and asters had been removed by sucking them into a micropipette inserted into the egg.

MATERIAL AND METHODS

The material used was the eggs of the heart-urchin, *Clypeaster japonicus*. This form was particularly chosen, because the egg is very transparent and the spindle and asters can be seen in the living condition fairly well.





Text-fig. 1 (*left*). Manipulation chamber. *E*, egg; *M*, microscope objective; *P*, micropipette; *W*, wall made of coverslip.
Text-fig. 2 (*right*). Relation between position of mitotic figure and cleavage plane.
a c, Successive stages of cleavage when the spindle is removed: *a*, before removal of the spindle; *b*, after removal; *c*, furrow appears at a predetermined position.
d f, Successive stages of cleavage when the mitotic figure is displaced by the removal of a part of egg protoplasm: *d*, before the displacement of the mitotic figure; *e*, after displacement astral rays of the lower aster are much elongated; *f*, furrow appears at a predetermined position.
In both series, cleavage planes are independent of shifted position of asters.

Eggs were fertilized in the ordinary way, and the fertilization membranes were removed by sucking the eggs into a fine pipette. When the eggs without the hyaline layer were needed, the eggs were washed three times with Ca-free sea water shortly after insemination. Before experimentation, the eggs were put into a glass chamber the wall of which tapered in a wedge shape (Text-fig. 1). A micropipette was inserted in a vertical direction into the egg. This method was more preferable than the ordinary hanging drop method because there was no fear of evaporation of the surrounding medium and the manipulation of the pipette was easier since its image was always in focus. The spindle or asters were sucked into a micropipette inserted into the egg in parallel with the spindle axis from the polar region. Pipettes with tips about 3 μ in diameters were chosen because they were sufficiently fine for the insertion and sufficiently large for the suction of the egg protoplasm. Observations were made from the horizontal direction with an ordinary microscope or a phase contrast microscope.[1]

[1] Professor K. Dan put to the author's disposal a phase contrast microscope with a condenser of a long focal length designed for microdissection experiment.

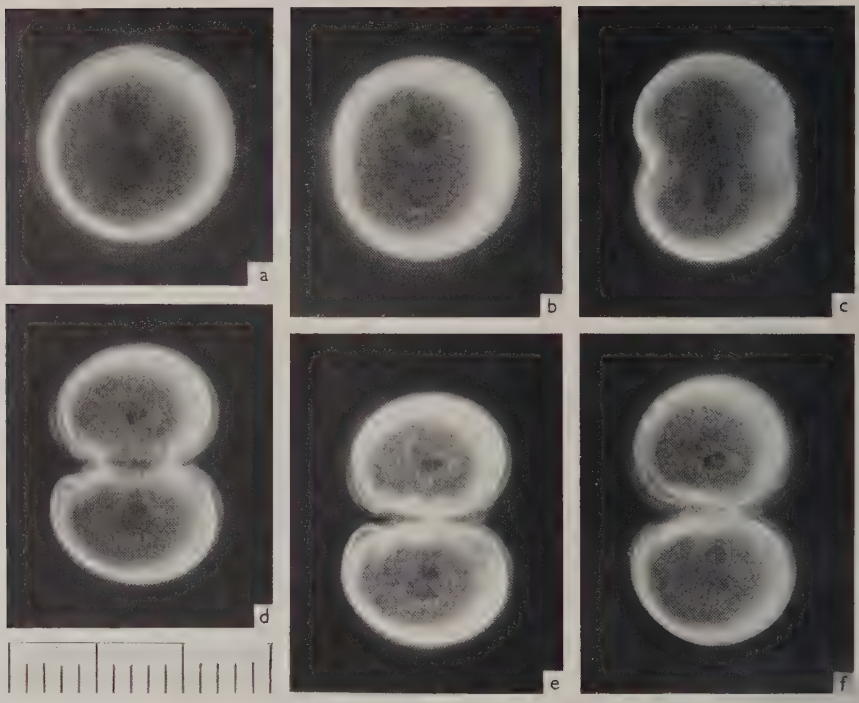


Fig. 1. Normal cleavage of *Clypeaster* egg in sea water by the phase contrast microscopy. *a*, 45.5 minutes after insemination; *b*, 49 minutes after insemination; *c*, 51 minutes after insemination; *d*, 53 minutes after insemination (dumb-bell stage); *e*, 54.5 minutes after insemination; *f*, 57 minutes after insemination. Fertilization membrane has been removed. The smallest division of the micrometer scale at the lower left corner is 10 μ. 27.5°C.

RESULTS

In *Clypeaster* eggs, the spindle and asters are visible in the living condition and they are more easily detectable when observed with a phase contrast microscope (Fig. 1).[1] Experiments were made in eggs both in sea water with the hyaline layer and in Ca-free sea water without the hyaline layer, although, in the latter case, there were tendencies that the eggs cytolysed from the point of the insertion of the pipette and that advance of furrowing in operated eggs was rather slow.

[1] Although the peripheral regions of eggs are not shown in photographs, details of the peripheral regions are seen as well as those of central ones in the original microscopic set-up.

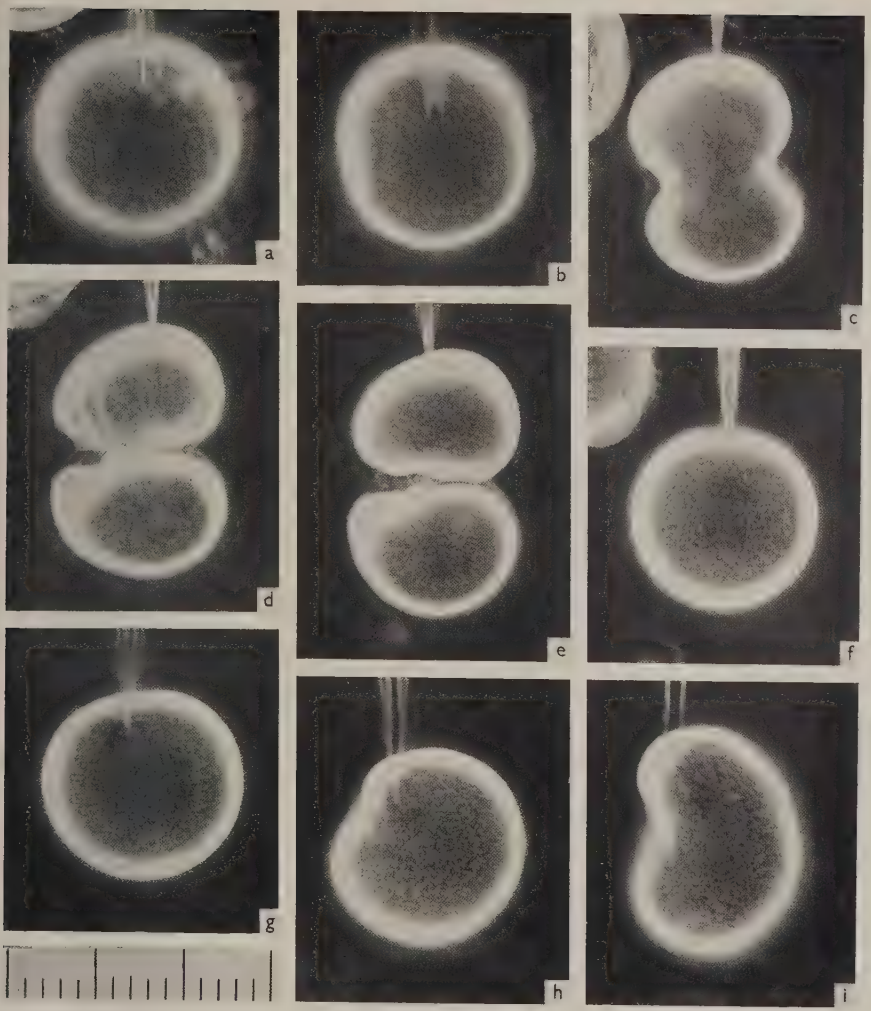


Fig. 2. Cleavage of *Clypeaster* eggs from which the spindles have been removed (P.C.M.). *a–e*, Successive stages of division without spindle in sea water. 27.5°C. *a*, 57 minutes after insemination. Micropipette has been just inserted into the egg. *b*, 58 minutes after insemination. Spindle is being removed. Dark area in the center of the egg is the remnant of the central region of the lower aster. *c*, 60.5 minutes after insemination. *d*, 64 minutes after insemination. *e*, 67 minutes after insemination. Cleavage is complete.
f–i, Successive stages of division without spindle in Ca-free sea water. 28°C. *f*, 47 minutes after insemination. Micropipette has been just inserted. *g*, 48 minutes after insemination. Spindle has been removed. *h*, 51 minutes after insemination. The furrow appears in the left side of the egg. *i*, 55 minutes after insemination. A sign of furrowing appears in the right side of the egg. The advance of furrowing stops at the stage of *i*, and the furrow recedes hereafter. The smallest division of the micrometer scale at the lower left corner is 10 μ.

When negative pressure was applied in the micropipette inserted into the egg, the spindle was sucked into the pipette (Fig. 2). The central regions of the asters were removed together with the spindle, and some of the astral rays were pulled into the pipette completely or partially. In this case, the peripheral regions of astral rays were still left in the periphery of the cell, thus some of astral rays were much elongated toward the tip of the pipette (Text-fig. 2*b*). When the spindle was completely removed at the dumb-bell stage of cytokinesis (Fig. 1*d*), most eggs continued furrowing, until finally they divided in two. If the spindle was removed during anaphase or early stage of furrowing (Fig. 2*b*), most eggs continued furrowing and some of them completed cleavage (Fig. 2*a–e*), while others stopped furrowing after a while and then the furrow receded (Fig. 2*f–i*). Cleavage plane was not modified by the removal of the spindle: if furrowing had already begun at the time of the removal the furrow continued to advance, and if the removal was made before the beginning of furrowing the furrow appeared in the presumptive position. The speed of furrowing of egg without spindle was slower than that of normal egg.

Cleavage plane was unmodified when the position of the mitotic figure was displaced during anaphase or later by the removal of a part of protoplasm (Text-fig. 2*d f*). Even when a fair portion of protoplasm was removed so that the cell surface surrounding it shrank, the position of the furrow remained unaltered, and thus unequal blastomeres were formed. From these facts, it may be concluded that the cleavage plane is already fixed on the cell surface in the above stages and bears no relation to the position of displaced mitotic figure.

Since the asters are small during metaphase, it was possible to remove the mitotic apparatus completely. In this case, neither the spindle nor asters were formed again; nevertheless the furrow appeared in a few eggs, while others showed no sign of cleavage. In one case, probably because of an incomplete removal of asters, one aster was formed in the egg which grew larger until it occupied the whole space in the egg. Even in this case, the furrow appeared in the presumptive cleavage plane. The fact above mentioned shows that the cell can initiate furrowing with neither spindle nor asters and the cleavage plane has been determined by the metaphase.

DISCUSSION

The foregoing experiments show that any theory of cleavage based on the mechanical push or pull of the astral rays on the surface of the cell or the

autonomous elongation of the spindle is inadequate, since cleavage may take place without mitotic apparatus and without relation to the displacement of the mitotic figure.

The close relation between position of the mitotic figure and the cleavage plane has been reported by many investigators. Although the capacity of the initiation of the furrow may be under the *influence* of the internal structures such as nucleus and mitotic apparatus, the motive force of furrowing is located in the cortex of the cell. The present experiments show that this capacity is established by the metaphase, which is earlier than the critical stage determined by Swann and Mitchison [11] in colchicine-treated eggs (mid-anaphase). There is a possibility that these critical stages determined experimentally tend to be judged later than it actually occurs, because under experimental conditions the cortex of the egg more or less suffers from injurious effects by colchicine in Swann and Mitchison's case [11] and by the insertion of the pipette in this author's experiment. On the other hand, the retardation of the speed of cleavage has been observed in both experiments. This fact can be explained partly by the insufficiency of the influence of the nucleus or mitotic apparatus on the cortex, partly by the injurious effects above mentioned. It can be considered that, the influence of the internal structures may continue after the metaphase although the influence accumulated by the time of metaphase is sufficient to *initiate* cleavage without a further cooperation of the mitotic apparatus.

According to Lorch [7], no cleavage (except "pathological cleavage") takes place before the re-formation of asters when the centrosphere is removed from sea urchin blastomere, and neither the formation of asters nor cleavage takes place when the nucleus is removed together with centrosphere. Since the removal of the centrosphere and nucleus was made in early stages of mitosis in her experiment, it seems that the capacity of furrow-formation was not established in the cortex, unless it was under the influence of reformed mitotic apparatus.

SUMMARY

1. Mitotic spindle and asters were removed from eggs of the heart urchin, *Clypeaster japonicus* by sucking them into a micropipette inserted into the egg.

2. Cleavage can take place when the spindle is removed during anaphase or later. In some eggs, the initiation of furrowing is seen when both spindle and asters have been removed during metaphase.

3. The position of the cleavage plane is unaltered in the above case as well

as when the mitotic figure is displaced by the removal of a part of egg protoplasm.

4. It is concluded that furrowing results from the active function of the cell cortex which, in turn, is under the influence of the internal structures (nucleus, spindle or asters).

The author acknowledges with thanks his indebtedness to Professors H. Kinosita and K. Dan for their valuable criticism and kind help in the preparation of the manuscript.

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BRIEF NOTES

CONNECTIONS BETWEEN MYOFIBRILS AND THE SARCOLEMMA AND BETWEEN ADJACENT MYOFIBRILS AS SEEN IN ELECTRON MICROGRAPHS OF VERTEBRATE STRIATED MUSCLE

M. KRAUSS and W. R. VAN ANTWERP

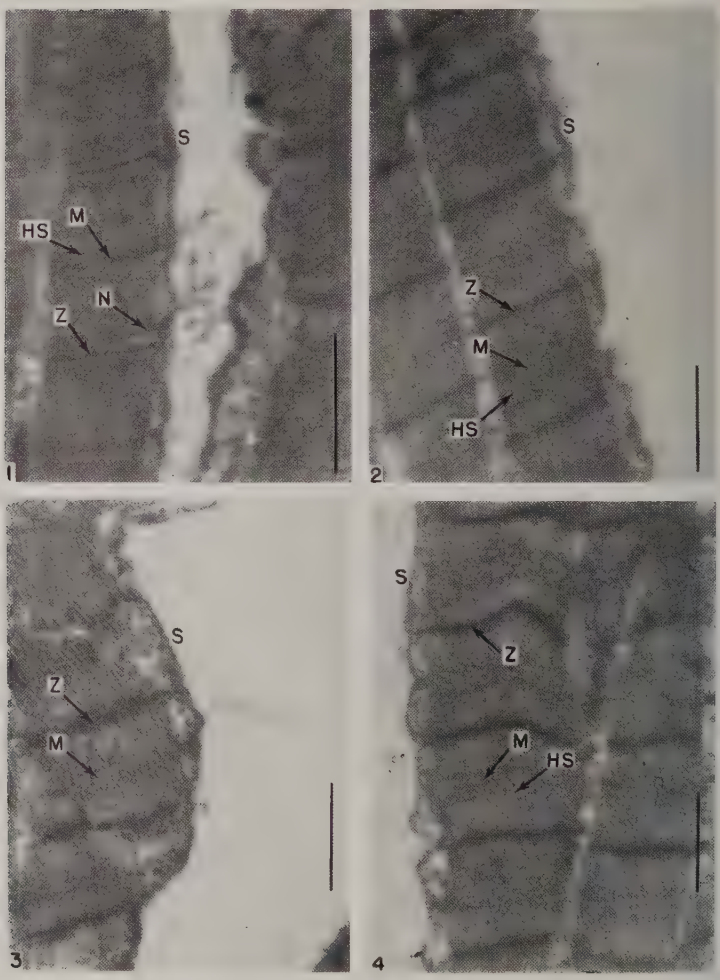
Biophysics Division and Test Division, Chemical Warfare Laboratories, Army Chemical Center, Maryland, U.S.A.

Received July 15, 1956

Recent electron microscope studies of vertebrate striated muscle [2, 4, 7] have confirmed and extended early observations made with the light microscope of connections between the inner layer of the sarcolemma and certain of the cross-striations of the myofibrils. Draper and Hodge [4] suggested that the innermost layer of the sarcolemma of toad muscle may be structurally continuous with the Z lines. Bennett and Porter [2] noted in sectioned fowl breast muscle that strands of sarcoplasm may occur on the inner surface of the sarcolemma in a pattern often corresponding to that of the Z lines, and less frequently and definitely to that of the M lines of adjacent myofibrils. Weinstein [7] observed on sarcolemmal fragments from fowl breast muscle similar strands whose distribution corresponded generally to that of the Z lines. Bennett and Porter and also Weinstein regard the connections between sarcolemma and myofibrils as tenuous and incapable of transmitting mechanical tension. Furthermore, these authors are in agreement that trans-sarcoplasmic connections between fibrils are inconstant and, in effect, do not provide for direct continuity of the Z lines across fibrils. This is in contrast to the view that Z lines extend continuously across the muscle fiber [4, 6].

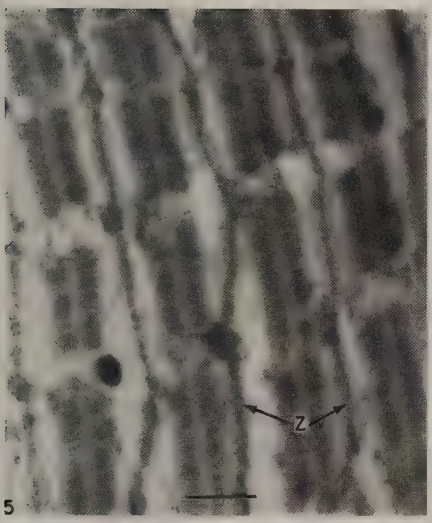
Myofibril-sarcolemma connections and trans-sarcoplasmic bridges between myofibrils obviously may be of importance in the functional activity of muscle fibers in addition to whatever structural or mechanical part they may have in fiber organization. The possibility that such connections may be involved in a conducting system within the fiber [1] is enhanced by the observation of A. V. Hill [5] that ordinary diffusion of a chemical substance is too slow to explain the rapidity of fiber response to membrane depolarization. Experiments with isolated fresh frog muscle fibers recently reported by Huxley and Taylor [6] are interpreted by them as indicating that the influence of membrane depolarization may be conducted to the interior of the fiber through the Z lines.

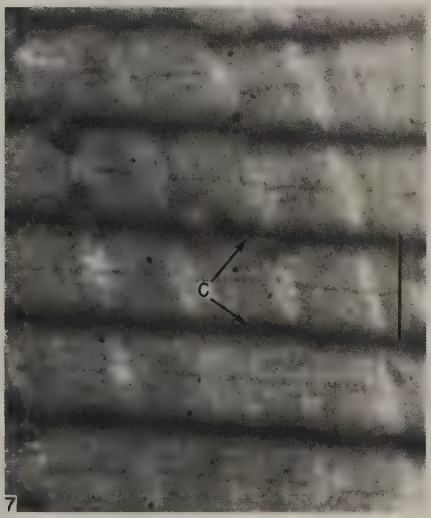
In the course of electron microscope studies of thin sections of vertebrate striated muscle from several species, we have observed well-defined connections between the sarcolemma and cross-striations of both the A and I bands of the myofibrils. All of our material was fixed in cold, buffered chrome–osmium solution [3], embedded in 9:1 butyl-methyl methacrylate mixture, sectioned with a Porter–Blum microtome

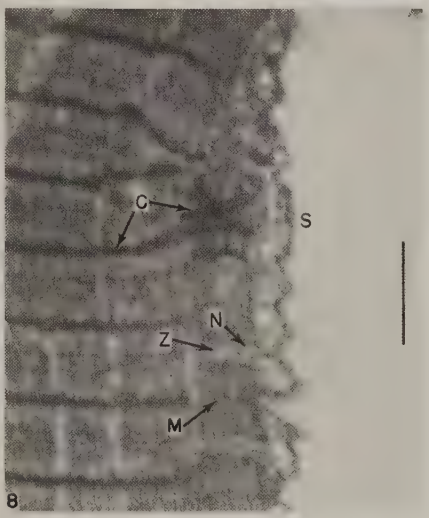


and examined with an RCA EMU 2A electron microscope. It can be seen in Fig. 1 to 4 and also in Fig. 8[1] that in addition to connections between the sarcolemma and the Z and M lines, faint but unmistakable bridges may also occur between the sarcolemma and the fibrils at other levels, notably in the region of the H sub-lines and N

[1] Figs. 1 through 4 and also 8 are frog sartorius; Figs. 5, 6 and 7 are goat rectus femoris. *C*, contraction bands; *HS*, H sub-lines; *M*, M line; *N*, N lines; *S*, sarcolemma; *Z*, Z line. The solid bar in each figure represents 1 micron.







lines of the A and I bands, respectively. In places where the sarcolemma is not closely applied to the myofibrils it frequently presents a scalloped appearance (Figs. 1–4, 8) with the indentations occurring opposite the Z and M lines of the fibrils.

The existence of connections between the sarcolemma and cross-striations in the A band as well as in the I band, raises the problem of how to account for selective

transmission of the influence of membrane depolarization through the I band [6]. If the Z line is in fact the main conduction pathway into the muscle fiber interior, we would expect to find some evidence of continuity between Z lines of adjacent myofibrils. In our preparations we have found considerable variability in Z-to-Z continuity—this is well illustrated by the figures. Very often it is difficult to determine if there is any sort of connection between Z lines of adjacent myofibrils. As a matter of fact, it is not infrequent to find adjacent myofibrils in which the Z lines are considerably out of phase as in Fig. 2. On the other hand, examples are by no means difficult to find in which definitive connections between Z lines are seen, and often there is little doubt but that these connections probably consist of actual extensions of Z line material across the inter-fibril sarcoplasm. This is clearly shown in Fig. 5 in which the I bands appear somewhat disorganized but the Z lines are intact and extend across several myofibrils. Fig. 6 shows a section in which three adjacent fibrils are split transversely through the I bands, allowing the Z line to lie free as a continuous thread across at least two and probably all three of the fibrils. In Fig. 7 and 8 are shown several fibrils in strong contraction with heavy contraction bands, which include the Z lines, running across them. These pictures leave no doubt that under some circumstances the Z lines do exhibit direct continuity between fibrils. At the present time we cannot say as much for any of the other cross-striations of the myofibril, nor can we yet define the conditions under which Z line continuity does occur.

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HISTOCHEMICAL DIFFERENTIATION OF NUCLEIC ACIDS BY MEANS OF INDUCED FLUORESCENCE

J. A. ARMSTRONG

National Institute for Medical Research, London, England

Received August 20, 1956

The use of fluorochrome dyes has been discussed in recent reviews on fluorescence microscopy [1, 3, 4]. Polychromatic fluorescence is sometimes induced by a single fluorochrome, but little is known about the mechanism or significance of these effects

and their application has been largely empirical. Some fluorescent derivatives of acridine have a strong affinity for certain tissues including many with a high nucleic acid content [5, 6, 8]; and *in vivo* administration of some amino-acridines produces monochromatic nuclear fluorescence [2, 7]. In this note initial results are reported from a systematic study of the fluorescence induced in fixed animal tissues by treatment with acridine orange R (2,8-*bis*-dimethylamino-5-phenylacridine hydrochloride, colour index number 792). The aim has been to control and investigate separately any steps in the technical procedure which appeared likely to influence the colour or intensity of the resulting fluorescence.

Normal rodent and ferret tissues used as test objects included liver, pancreas, spleen, stomach, trachea, uterus, gonads, kidney and spinal cord. Small pieces, after brief fixation in Carnoy's fluid, were sectioned in paraffin at 3 μ. Adjacent sections were mounted separately on thin glass slides. Routinely, several sections of each tissue were taken to distilled water and then placed for 15 min. into separate solutions of acetate or veronal acetate buffer, covering the range of pH 0.6 to pH 9.1. After treatment for 30 min. with correspondingly buffered solutions of acridine orange (concentration 1 in 2000) the sections were immersed for at least 15 min. in fresh buffer. Finally they were mounted in a drop of buffer and sealed with wax. Departures from this routine were designed to show the effects of different fixatives or of different concentrations of acridine orange. The semi-permanent preparations could be examined at leisure on the fluorescence microscope, the colour and intensity of fluorescence shown by the different cellular components being tabulated at each value over the pH range. The source of activating light was a high pressure mercury vapour lamp and illumination of the object by incident light was achieved by means of a dark ground condenser. By choice of filters the activation was with either long wave ultraviolet (3650 Å) or with visible blue light both being used on each specimen. All tissues were examined by ordinary microscopy for comparison, after being stained with haematoxylin and eosin, methyl green-pyronin, Feulgen's stain or the periodic acid–Schiff reaction.

It was at once apparent that the amount of acridine orange taken up by the sections from solution increased with rising pH. Above pH 6 all sections exhibited strong red fluorescence with virtually no tissue selectivity. Below pH 1.5 on the other hand, dye take up was minimal and little more than diffuse blue fluorescence could be elicited. At intermediate values the various tissues, cells and cell components showed marked differences in their affinity for acridine orange, and there was consequently a sharp differential fluorescence of the cellular elements. The most remarkable results occurred in the pH range 3.6 to 5.2, when a wide range of spectral colours were seen together in one section. With more dilute solutions of acridine orange (1 in 20,000 or 1 in 100,000) longer staining was required for the development of maximum intensity of differential fluorescence, but the end result was the same. Of particular interest was the characteristic fluorescence of nuclear and cytoplasmic basophil elements, with a sharp and stable colour differentiation of structures containing desoxyribonucleic acid (DNA) or ribonucleic acid (RNA). With blue light activation, nuclear chromatin fluoresced bright yellow, whilst cytoplasmic RNA was flame red in colour. The corresponding fluorescent colours with ultraviolet activation were greenish-yellow and crimson red. Intense red fluorescence was evident in the basal cytoplasm of the pancreatic exocrine cells (Figs. 1 and 2) and in the peptic cells of the gastric

mucosa (Fig. 3), in the Nissl substance of nerve cells and in the cytoplasmic basophil bodies of the liver parenchyma (Fig. 4). Red fluorescence of slightly lower intensity was seen in many cells, for example in the columnar uterine epithelium (Fig. 5), in the tracheal glands, and in basophilic elements of the splenic pulp (Fig. 6). At high magnification it was seen that the nucleolus, at least in certain epithelial cells and neurones, is a red fluorescing body encircled by "associated chromatin" showing bright yellow fluorescence. The nature of the fluorescent structures was invariably confirmed by the Feulgen reaction and by methyl green–pyronin staining. It appeared therefore that these observations might form the basis of a histochemical procedure for the localisation and identification of the nucleic acids. Similar results were achieved with tissues fixed in Zenker's fluid or in corrosive-acetic; after Bouin fixation however, there was no distinctive fluorescence of cytoplasmic RNA.

Pretreatment with ribonuclease[1] prevented the red fluorescence of cytoplasmic basophil structures, leaving nuclear chromatin unaffected. Similarly, the yellow fluorescence of chromatin was selectively prevented by pretreatment with desoxyribonuclease.[1] Moreover, smears prepared from purified DNA and RNA, treated with Carnoy's fixative and stained in the usual manner, acquired yellow and red fluorescence respectively. Red fluorescence of mast cell granules and of cartilage matrix, and a yellow fluorescence of the vascular elastic laminae provided the only examples of "false positive" reactions in the relevant pH range. They differed from the nucleic acids, however, by retaining their colour at much lower pH values and by remaining unaffected by pretreatment with ribonuclease or desoxyribonuclease. Powdered acridine orange, as obtained from the manufacturer,[2] contained traces of impurities some of which fluoresced in ultraviolet light, but removal of these by electrophoresis did not detectably alter the fluorochroming properties of the dye.

Antibacterial mechanisms have aroused recent interest in the physicochemical interaction of acridines and the nucleic acids [9]. The complexes formed by acridine orange with DNA and RNA in fixed tissues are now shown to have different fluorescent characteristics of a stable nature, which allows a promising and simple alternative to basic dye mixtures for the differentiation of the nucleic acids in tissue sections. The sensitivity of the method for cytopathological purposes and its application for quantitative work are being investigated.

[1] Kindly supplied by Professor J. N. Davidson, Department of Biochemistry, Glasgow University.

[2] Messrs. Hopkin & Williams Ltd., Chadwell Heath, Essex, England.

Fig. 1. Pancreas of a ferret, showing yellow fluorescence of nuclear chromatin and red fluorescence of the basophil cytoplasm in the exocrine cells. Magnification ×340.

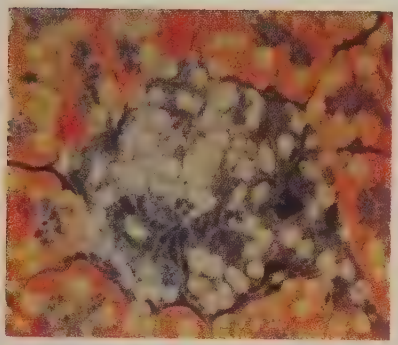
Fig. 2. Higher power view of secretory cells in the wall of a pancreatic duct in the ferret, showing the mainly basal location of cytoplasmic red fluorescence. Magnification ×470.

Fig. 3. Mucosa of ferret stomach. Note the red fluorescing cytoplasm of peptic cells. Magnification ×340.

Fig. 4. Rat liver. Magnification ×340.

Fig. 5. Uterine epithelium of a young unmated rat. Magnification ×270.

Fig. 6. A collection of cells with red fluorescing basophil cytoplasm in the splenic pulp of a ferret. Magnification ×270.



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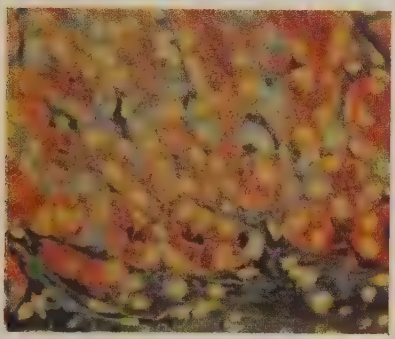

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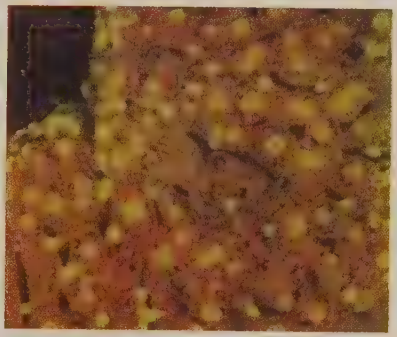

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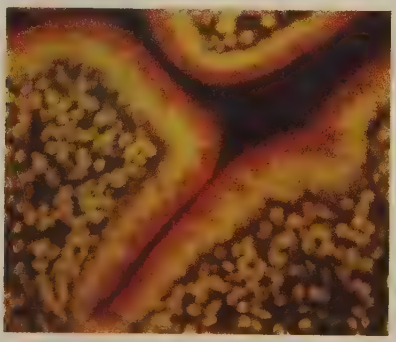

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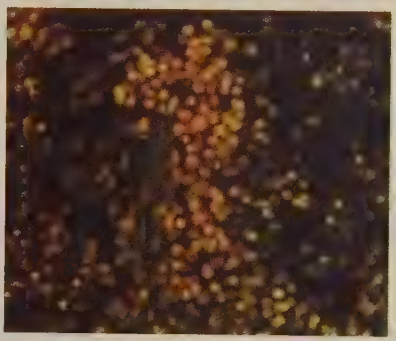

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STAINING MITOCHONDRIA IN FROZEN-DRIED TISSUES[1]

J. P. CHANG[2]

Department of Pathology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas, U.S.A.

Received September 21, 1956

It is well known that the cytologic details and chemical constituents of cells are more faithfully preserved by freeze-drying than by chemical fixation. For this reason, frozen-dried tissues have been used extensively for a variety of histochemical and microchemical tests. So far as the author is aware, no standard technique for staining mitochondria in frozen-dried tissue has been established. The staining methods used most often to demonstrate mitochondria in chemically fixed tissues have been either the original or some modification of the several procedures of Altmann [1], Benda [3] and Regaud [7]. These methods may be non-specific, capricious and tedious, and may require considerable manipulative experience [2, 5]. Herein is presented a technique of staining mitochondria in frozen-dried tissues which is simple and reliable, and thus void of the defects of the classical methods.

A successful staining reaction can be obtained only with properly frozen-dried tissue, in which the cytoplasm, when stained with haematoxylin and eosin, appears homogeneous, with well defined granules, and the architecture of the tissue is unaltered (Fig. 1). Vacuolization, ice crystal formation and cytoplasmic granule coalescence indicate poorly frozen-dried tissue, which will not stain satisfactorily. In a freeze-dry run, every piece of tissue must be handled individually; consequently,

[1] This research is supported in part by a grant (PHS No. C-1913) from the National Institutes of Health, U.S. Public Health Service.

[2] The author gratefully acknowledges the technical assistance of Miss Velma Yemoto.

each piece may differ slightly from all the others in the same run. Before proceeding with the staining, therefore, it is advisable to make an haematoxylin and eosin stained slide of each piece of tissue to be sure it has been properly frozen-dried.

Another factor which affects the staining quality is the thickness of paraffin sections; the thinner the section, the better the staining reaction. The author generally uses one micron sections, since more thickness gives undesirable background staining.

Technique. Mix 4 to 6 cc of anilin oil with 100 cc of distilled water. With a mechanical stirrer, stir the mixture continuously for 6 hours at toom temperature. Filter off the excess anilin oil. Mix 100 cc of the filtrate (saturated anilin water) with 10 grams of acid fuchsin (certified National Anilin dye—dye content 57 per cent) and 2 drops of glacial acetic acid. Heat the mixture in a 60°C oven for 4 hours, in a well stoppered glass bottle. Allow the dye to stand at room temperature for at least a week for aging. Filter the dye solution before each usage. This anilin fuchsin solution may be stored at room temperature in a well stoppered amber bottle and used repeatedly for 6 to 10 weeks, with good results.

The tissue may be prepared with the apparatus described by Stowell [8], Freed [4], Moberger *et al.* [6] or others.

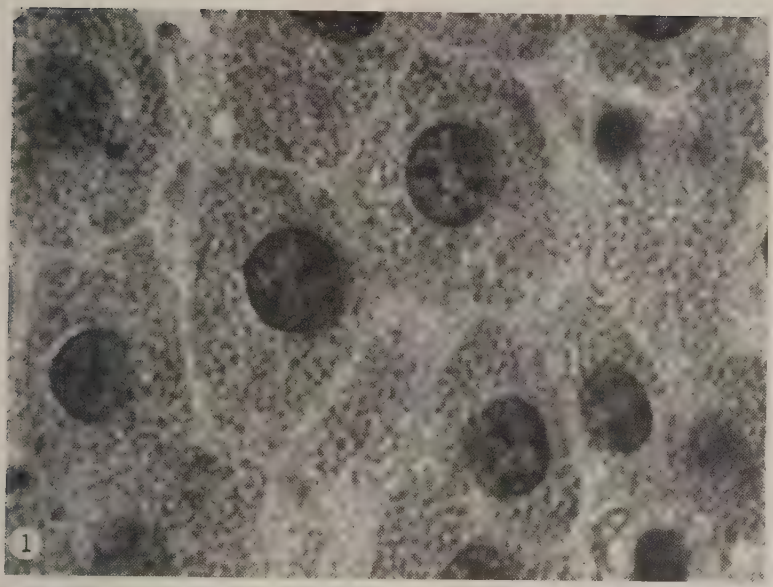
Place one drop of 3 per cent formalin containing 1 per cent calcium chloride upon an albuminized slide. Float one micron sections onto the formalin solution and place the slide on a slide warmer. (Poorly frozen-dried tissues disintegrate in formalin solution!) As soon as the sections are flattened, quickly drain off the excess formalin and return the slides to the slide warmer for drying. Immerse the nondeparaffinized slides for 24 hours directly in 5 per cent potassium dichromate containing 1 per cent calcium chloride at room temperature, then wash in running water for 1½ hours and air dry. Pass the slides rapidly through xylene, alcohol series to distilled water. Stain for 8 to 15 minutes (depending upon the kind of tissue) in anilin fuchsin solution prepared according to the above described method and preheated at 60°C. Rinse briefly in distilled water. Differentiate in saturated anilin water for 2 to 5 minutes. Rinse thoroughly in distilled water. Counterstain for one minute with 1 per cent aqueous methyl green solution. Pass through 95 per cent to 100 per cent alcohol and xylene and mount in Permount (Fig. 2).

The finished slide shows that the mitochondria are stained bright red against a slightly pink background; the nuclei are stained faintly green; the erythrocytes, dark red; and collagen, red to purplish.

Our preliminary experiments have shown that this method may also be employed for staining tissues fixed by alcohol freezing-substitution and by 10 per cent formalin containing 1 per cent calcium chloride.

This technique is highly reproducible, provided the tissue is properly frozen-dried and the section is thin. It may be carried out by persons who have had no previous experience in its use. Liver, kidney, intestine and pancreas tissues of mice, rats, guinea pigs and human beings have been successfully stained with the method. The colors have not faded during eight months in storage. The results of its application to the study of mitochondria in tumor tissues will be published at a later date.

Fig. 1. Properly frozen-dried normal mouse liver, haematoxylin and eosin stain, oil immersion.
Fig. 2. Mitochondria in frozen-dried mouse liver, oil immersion.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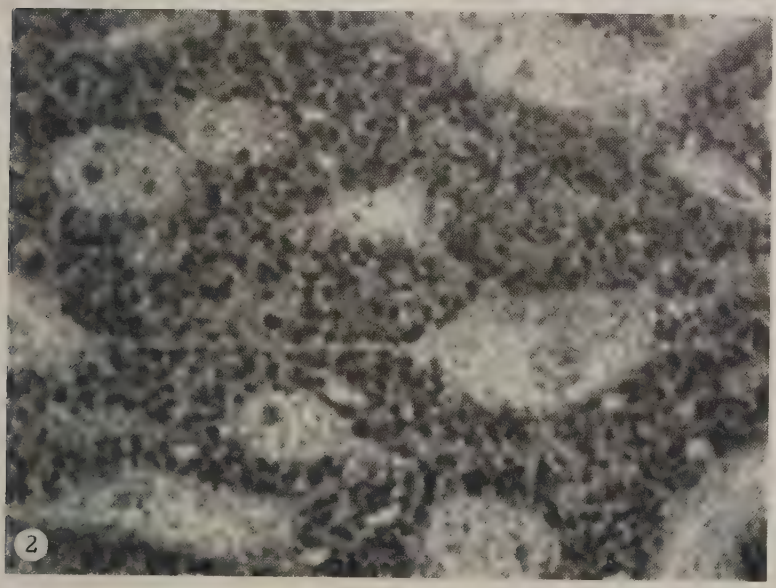
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IS THE PARENCHYMA OR THE STROMA OF AN ORGAN THE PRIMARY TARGET OF X-IRRADIATION?

T. S. ARGYRIS and H. B. CHASE

Department of Zoology, Syracuse University, Syracuse, N.Y., and Department of Biology, Brown University, Providence, R.I., U.S.A.

Received September, 28, 1956

Whether the parenchyma cells, or the supportive connective tissue cells of an organ, are the primary targets of X-irradiation is still an unanswered question. The following experiments with mouse skin irradiated at different stages of the hair growth cycle have been undertaken in an attempt to add more information to this problem.

For an understanding of the experimental design some introductory remarks are advisable. Mouse skin undergoes cycles of growth and rest. Growth of the skin may be initiated by plucking of the resting follicles [4]. Following plucking, the resting hair follicles undergo proliferative activity in the germ region and the follicle begins to grow into the subcutaneous tissue. The cells in the bulb area envelop the papilla and keratinization begins. Associated with these changes in the hair follicles, the dermis and subcutaneous tissues increase greatly in size and vascularity [4]. Continued mitotic activity in the external root sheath and in the germ region enables the follicle to attain its maximum length and produce its hair shaft. The differentiation of new follicles from resting ones is largely completed within 5–6 days after plucking. For the remainder of the 12–13 days of the growth phase of the cycle the already differentiated follicles primarily elaborate hair. These follicles may be called growing hair follicles, whereas those in the early formative phases of the hair cycle may be called differentiating follicles. After completion of the formation of the hair shaft the follicles are transformed into resting ones and the cycle is completed.

Previously it has been shown that differentiating or growing follicles [1, 2] are more sensitive to X-irradiations of 5000 r than are resting ones. After irradiation, differentiating follicles show pronounced microscopic damage within 2–3 days and further development is stopped. Grossly, this is evident within 2–5 days after treatment, depending on the stage of differentiation at which the follicles are irradiated. In

growing follicles, damage is apparent microscopically within 3 days, and hair loss occurs 4–6 days after irradiation. On the other hand, resting follicles do not show microscopic changes for at least 7 days, and hair loss is not evident until 12–21 days after irradiation [1]. Therefore, if resting follicles could be placed in skin in the growth phase of the hair cycle, and the response of these follicles to X-irradiation could be determined, an answer to our question might be obtained. Under such conditions, not only the plucked follicles will have undergone changes but also the dermis and subcutis will have enlarged and their vascular supply increased. By determining the response to X-irradiation of the resting hair follicles in this "growing" environment, and comparing it to the response of resting hair follicles in their normal environment (resting skin), a measure of the influence of the environment on the response of the resting follicles to X-irradiation can be obtained.

The resting hairs on the dorsum of 7 C_{57} black mice were plucked being careful to leave a few resting follicles untouched. Three days after plucking, a time at which the plucked follicles and the supportive tissue had undergone growth changes, the animals were irradiated with 5000 r. A Picker–Waite, 200 KV therapy machine was used at 20 ma. Irradiation was done at a distance of 17 cm with no additional filtration, the dose rate being 1000 r/min. A circular area of 8 mm in diameter was irradiated. The rest of the mouse was covered with appropriate lead shielding. The irradiated areas were checked daily under a binocular microscope and the effect of the X-irradiation on the resting follicles and on the surrounding growing follicles and surface of the skin noted.

Within 2 days after irradiation development of the differentiating follicles is stopped. By 7 days the surface of the wound is scaly. Soon sloughing becomes apparent and by 12–14 days the center of the irradiated area is pitted indicating the presence of an ulcer. During this time the resting follicles, embedded in this damaged "growing" environment, arranged singly or in small groups, are completely unaffected. Hair loss is not apparent until 12–16 days.

To be certain that partial hair loss did not occur another group of 6 C_{57} black mice were irradiated as already indicated. Immediately after irradiation, the treated area was marked with a solution of eosin and the number of resting hairs in the irradiated area were counted. These hairs were again counted at 7, 10 and 12 days. As before, hair loss did not occur until 12–14 days, the time at which it would have occurred if the resting follicles were in their own environment.

Current experiments are extending these findings to include observations on the response to X-irradiation of resting follicles present in skin at a variety of growth stages of the hair cycle, since the dermis and subcutis change constantly during the first 6–7 days of the hair cycle [3]. To date, in no case has the environment affected the response of the resting follicles. Always, irrespective of the stage of growth of the skin at the time of irradiation, the resting follicles do not lose their hairs until at least 12–14 days after irradiation. Histological studies are also being done to ascertain whether or not the microscopic changes in such resting follicles are the same as they are in resting follicles irradiated in their normal environment. The sections so far studied confirm our belief that the microscopic changes are the same.

The conclusion is that resting follicles embedded in a growing environment, respond just as they would if they were left in their own connective tissue environment. Environmental changes, as increased blood supply, do not affect the response of the

resting follicles to irradiation. The conclusion is not yet unwarranted, however, that growing follicles if placed in a foreign environment (resting skin) will also be unaffected by the surrounding supportive tissue in their response to X-irradiation. Since resting hair follicles [1] are relatively radio-resistant epithelial tissues it might be speculated that the epithelial cells of radio-resistant organs in general are the primary targets of irradiation, and that these cells govern their response to irradiation, and not the supportive tissue in which they are embedded. The complementary speculation, however, that radiosensitive tissues in general respond to irradiation independently of their environment is not justifiable. Experiments to answer this question are currently being undertaken.

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L'INDUCTION PAR ORGANISATEURS DÉVITALISÉS

R. LALLIER

Institut de Biologie Physico-Chimique, Paris

Reçu le 12 octobre, 1956

Dès 1932, des recherches [1, 3] ont établi que l'organisateur tué par des traitements les plus variés (chaleur, congélation, dessication, action de l'alcool) conserve ses propriétés inductrices. En examinant la résistance des propriétés inductrices à l'action de différents traitements nous avons montré que le formol inactive les propriétés inductrices de l'organisateur [4]. Les recherches récentes de Yamada et Takata [6] montrant l'inactivation des propriétés inductrices du rein et de ses fractions par des enzymes protéolytiques tels que la trypsine et la chymotrypsine mettent l'accent sur la participation éventuelle de substances protéiques au cours de l'induction. L'inactivation des propriétés inductrices de l'organisateur par le formol peut ainsi se concevoir en raison de l'action de cette substance sur les protéines. A cet égard il est intéressant de rappeller ici que le formol inactive les toxines microbiennes en les transformant en toxoïdes dépourvus de propriétés toxiques. L'étude de ces phénomènes peut apporter quelques précisions sur le mode d'action du formol sur les protéines. D'une façon générale les protéines sont dénaturées dans les organisateurs tués. Nous remarquerons toutefois que certaines protéines engagées dans des formations complexes lipidiques et glucidiques peuvent, dans une certaine mesure, échapper à la dénaturation. La dénaturation des protéines entrainant en outre le démasquage de nouveaux groupes fonctionnels, leur présence conditionne à son tour la formation de nouveaux complexes entre ces protéines et d'autres constituants cellulaires. Afin de s'orienter dans les phénomènes multiples provoqués par l'action des traitements

tuant l'organisateur, il apparaît nécessaire d'utiliser des agents se signalant par une action caractéristique. Les agents qui, outre les effets généraux et complexes entrainés par la fixation des tissus, exercent une action définie, telle par exemple le blocage de groupes réactionnels déterminés, répondent à ces conditions. En examinant l'activité inductrice d'organisateurs ainsi traités, nous pourrons tracer une relation entre la perte ou la conservation de cette activité et le blocage de groupements donnés.

Dans ces expériences nous examinerons à cet effet d'une part, les propriétés d'organisateurs tués et colorés par le bleu Evans, colorant acide polysulfonique présentant une affinité marquée pour les groupements basiques des protéines et d'autre part, les propriétés d'organisateurs tués dont les groupes sulfhydriles sont oxydés par l'iode.

Les expériences ont été faites au cours de l'été 1956, sur les embryons de l'amphibien *Triton alpestris*. Les organisateurs sont constitués par les lèvres blastoporales prélevées sur de jeunes gastrulas. Une heure après le prélèvement, ces organisateurs bien cicatrisés sont traités de la façon suivante. Dans une première série d'expériences, les organisateurs préalablement fixés par un séjour de deux heures dans l'alcool à 95°C, sont ensuite colorés en les mettant pendant deux heures dans une solution de bleu Evans à 1/1000 dans le tampon acétate de sodium acide acétique (0,04 *M*) à pH 4. En milieu acide les protéines fixent davantage de bleu Evans [5]. Les organisateurs sont fortement colorés en bleu foncé dans toute leur masse, ce que l'on vérifie aisément en les examinant après les avoir sectionnés en deux. Pour éliminer l'excès de colorant et le tampon acide qui les imprègne, les organisateurs sont lavés avec du tampon phosphate à pH 7, pendant deux heures et ensuite avec la solution de Holtfreter, également pendant deux heures. Au cours de ces lavages les solutions sont renouvelées plusieurs fois. Des organisateurs traités par le tampon acéate de sodium–acide acétique à pH 4 seul, sans bleu Evans, et lavés comme précédemment, sont utilisés comme témoins. Les organisateurs traités et témoins sont insérés dans le blastocèle de jeunes gastrulas.

Dans une seconde série d'expériences, les groupes sulfhydriles des organisateurs sont oxydés en mettant les organisateurs pendant deux heures dans une solution d'iode (0,06 *M*) dans l'iodure de potassium. On ajoute juste la quantité d'iodure nécessaire pour dissoudre l'iode. Le pH de la solution est environ 6. Après ce traitement les organisateurs sont intensément colorés en brun dans toute leur masse. Leur consistance est dure et friable. On élimine l'iode qui les imprègne en les lavant pendant deux heures dans une solution d'hyposulfite de soude à 4/1000, que l'on renouvelle plusieurs fois. Après ces lavages la coloration brune a complètement disparu dans toute la masse de l'organisateur et celui-ci a perdu sa dureté et sa fragilité. Le tétrathionate de sodium formé au cours du lavage à l'hyposulfite de sodium est éliminé en lavant les organisateurs pendant deux heures avec une solution de Holtfreter fréquemment renouvelée. Les organisateurs sont ensuite insérés dans le blastocèle de jeunes gastrulas.

Les embryons opérés sont cultivés stérilement dans la solution de Holtfreter diluée à 1/10. Après 75 heures ils sont fixés dans le liquide de Smith puis coupés à 10 μ et colorés par la méthode de double coloration au bleu de Célestine, éosine.

L'oxydation des groupes sulfhydriles dans les organisateurs traités par l'iode a été vérifiée par le test au nitroprussiate de sodium. Ce test est négatif dans les organisa-

teurs traités, indiquant que les groupes sulfhydriles sont oxydés. Les lavages à l'hyposulfite n'entraînent pas la reformation des groupes sulfhydriles aux dépens de groupes sulfhydriles oxydés. Des organisateurs traités insérés dans le blastocèle et laissés pendant 3 jours au contact des tissus de l'hôte donnent également une réaction négative avec le nitroprussiate, indiquant que dans ces conditions les groupes sulfhydriles ne se reforment pas dans l'organisateur tué. Dans les organisateurs témoins, non traités par l'iode, la réaction au nitroprussiate est fortement positive indiquant ainsi la présence de nombreux groupes sulfhydriles. Outre son action oxydante, l'iode peut se fixer sur la tyrosine et l'histidine, ainsi que sur des lipides non saturés. Avec les acides aminés, la fixation de l'iode est favorisée par un pH alcalin. La tyrosine donne une coloration brune avec le réactif de Millon, cette coloration disparaît lorsque la tyrosine est iodée. Avec le test de Millon exécuté selon la technique de Bensley et Gersh [2], les organisateurs traités par l'iode développent une coloration brune comparable à celle observée dans les organisateurs témoins. Ces résultats indiquent que dans les conditions expérimentales utilisées les groupes tyrosine ne fixent pas l'iode de façon appréciable.

Résultats.—Les 14 embryons examinés, greffés avec un organisateur tué et coloré par le bleu Evans présentent une volumineuse vésicule neurale dans 12 cas et un épaississement épiblastique important dans 2 cas. La coloration bleue des organisateurs est bien visible sur les coupes. Le colorant ne passe pas dans les tissus de l'hôte situés au contact du greffon.

Les 9 embryons témoins examinés traités avec le tampon acétate–acide acétique à pH 4 seul, sans colorant, présentent une volumineuse vésicule neurale induite dans 7 cas et un épaissement épiblastique important dans 2 cas.

L'examen des 14 embryons greffés avec un organisateur traité par l'iode, montre que 10 d'entre-eux présentent une vésicule neurale volumineuse et les 4 autres un épaississement épiblastique au contact du greffon.

Dans tous les cas examinés les inductions sont situées dans la région troncale moyenne de l'embryon, sur la ligne médio-ventale. Le volume et la nature des structures neurales induites sont comparables.

Ces résultats font ressortir que les organisateurs tués conservent leurs propriétés inductrices malgré la formation de complexes entre leurs protéines d'une part et un colorant acide polysulfonique, le bleu Evans, d'autre part. La coloration des organisateurs tués a lieu dans un tampon à pH 4. Dans ces conditions la majeure partie des groupements basiques des protéines sont combinés avec les groupements sulfoniques acides du bleu Evans.

L'étude de l'activité inductrice des organisateurs traités par l'iode montre d'autre part que l'oxydation des groupes sulfhydriles par cet agent n'altère pas les propriétés inductrices de l'organisateur.

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VERGLEICHENDE ELEKTRONENMIKROSKOPISCHE BEOBACHTUNGEN ZUR INTRAZELLULÄREN EISENABLAGERUNG[1]

H. SCHULZ[2]

Anatomisches Institut des Karolinska Institutes, Stockholm, Schweden

Eingegangen am. 4. Oktober 1956

Elektronenmikroskopische Schnittuntersuchungen an Lungen- und Lebergewebe der Ratte zeigten in den Alveolarepithelien sowie in den Kupfferschen Sternzellen der Leber osmiophile Körper, die verschieden große, für Elektronen nicht oder schwer durchstrahlbare Granula enthalten, von denen angenommen wird, daß sie Hämosiderin bzw. Ferritin darstellen.

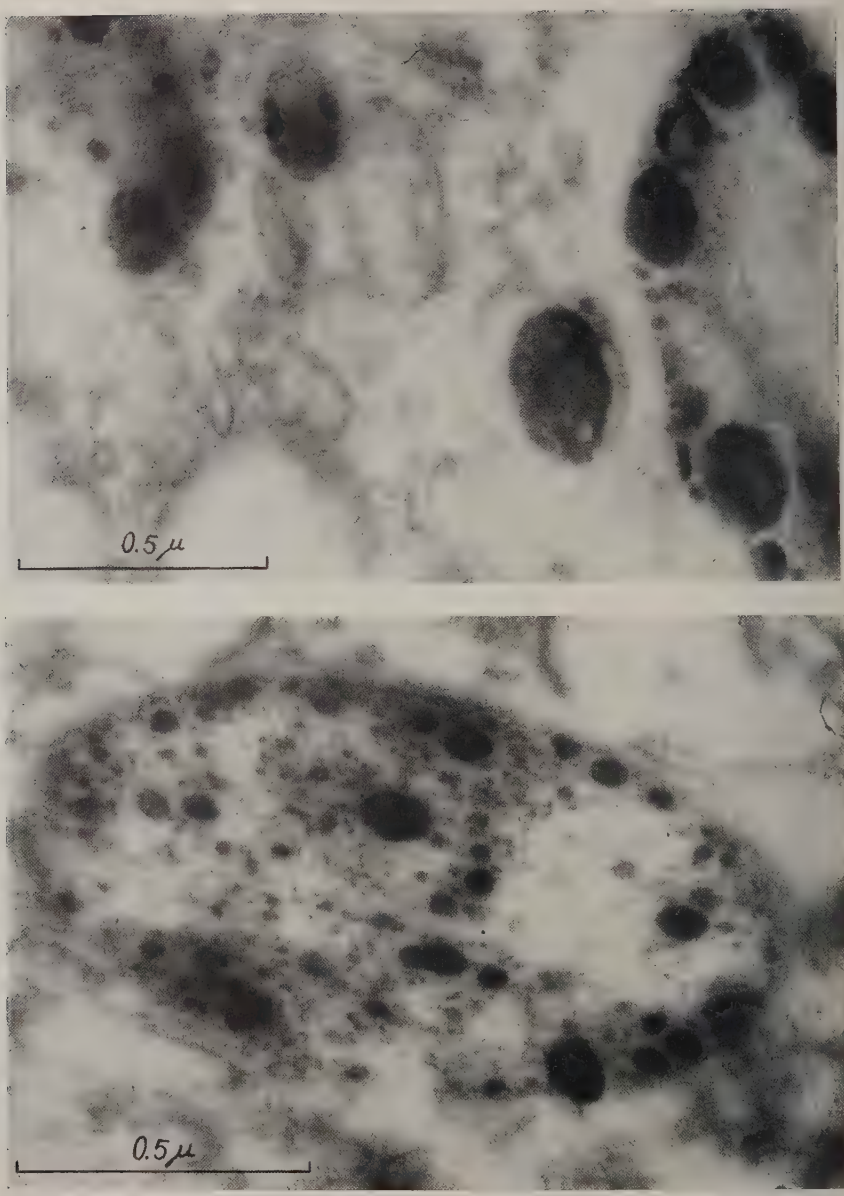
In einer ersten Untersuchungsserie beobachteten wir das Verhalten der Alveolarzellen beim experimentellen Lungenödem [5]. Im Cytoplasma der Alveolarepithelien liessen sich zahlreiche, im Längsdurchmesser 0,2 bis 1,4 μ große ovale osmiophile Körper nachweisen (Abb. 1), die in Gruppen vorkommen und meist in der Nähe des Zellkerns liegen. Einige osmiophile Körper haben eine Membran, andere sind gegen die Grundsubstanz des Cytoplasmas nur scharf begrenzt. Die Körper enthalten sehr kontraststarke runde oder ovale Körnchen, die elektronenoptisch eben noch eine unterschiedliche Dichte erkennen lassen. Die Körnchengröße variiert sehr. Der Durchmesser der am häufigsten anzutreffenden Körner beträgt 0,17–0,32 μ. In einigen osmiophilen Körpern zeigen die kontrastreichen Granula eine auffallende randständige Lagerung (Abb. 1).

In einer zweiten Untersuchungsserie beschäftigten wir uns vor allem mit der Frage der Veränderungen an den Mitochondrien der Leberzellen nach Behandlung mit DL-thyroxin [6]. Als Nebenbefund sahen wir im Cytoplasma der Kupfferschen Sternzellen ähnliche Gebilde wie in den Alveolarepithelien. Es handelt sich hier um 0,5 2,4 μ große ovale osmiophile Körper, die im Gegensatz zu den osmiophilen Körpern des Alveolarepithels eine relativ große Anzahl von kontrastreichen Körnchen enthalten (Abb. 2). Die kleineren Granula sind im Durchmesser 80–240 Å groß. Häufig sind viele kleine Körnchen eng aneinandergelagert.

Mit unseren Beobachtungen möchten wir hervorheben, daß die organischen Eisenverbindungen nicht als freie Substanzen in der Zelle vorkommen, sondern in Form kontrastreicher Granula in den osmiophilen Körpern gespeichert sind. Für die Annahme, daß die kontrastreichen Granula Eisen enthalten, bestehen verschiedene Gründe. In eigenen elektronenoptischen Untersuchungen der Lunge bei Mitralstenose [4] fanden wir bereits in den Alveolarepithelien osmiophile Körper mit einem eigentümlichen Entwicklungszyklus. Diese osmiophilen Körper enthielten kontrastreiche Körnchen, welche mit den Körnchen in den osmiophilen Körpern der Alveolarzellen der Rattenlunge übereinstimmen. Die damals zum Vergleich durchgeführte lichtmikroskopische Untersuchung zeigte eine positive Berlinerblaureaktion, so daß man

[1] Herrn Dr. F. S. Sjöstrand, Prosektor des Anatomischen Institutes, danke ich sehr für die Ermöglichung meiner Arbeit in seinem Laboratorium.

[2] Assistent des Pathologishen Institutes der Medizinishen Akademie in Düsseldorf.


0.5 μ
0.5 μ

annehmen kann, daß die Körnchen durch Hämosiderinablagerungen einschließlich ihrer Eiweißlipoidkomponenten zustande kommen. Auch unter physiologischen Bedingungen wird das Eisen an die osmiophilen Körper adsorbiert, wie durch die Untersuchungen von Hirsch [1] bestätigt werden konnte. Osmiophile peribiliäre Körper mit einem feingranulären Gefüge von unterschiedlicher Körnelung beschreiben Palade und Siekevitz [3] in elektronenmikroskopischen Untersuchungen der normalen Leber. Obgleich die chemische Natur der Körnchen unbekannt ist, vermuteten Palade und Siekevitz in den Körnchen eine Metallverbindung. Vitaminmangelpigmente möchten wir für die Bildung der Körnchen nicht verantwortlich machen, da in elektronenmikroskopischen Beobachtungen von Lindner [2], Sheldon und Zetterqvist [7] die Mangelpigmente der Vitamine A und E als Mitochondrienprodukte herausgestellt worden sind und die Pigmente submikroskopisch eine andere Struktur aufweisen.

Zur Speicherung des Eisens in der Zelle haben wir die Vorstellung, daß sowohl das Hämosiderin als auch das Ferritin an die osmiophilen Körper lokalisiert ist.

Weitere Untersuchungen zur Bestätigung unserer Annahme sind noch erforderlich.

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Abb. 1 (*oben*). Ausschnitt einer Alveolarepithelzelle der Rattenlunge. Osmiophile Körper mit kontrastreichen Granula. Fixierung nach Sjöstrand. Aufnahme mit dem RCA EMU 2c Elektronenmikroskop. Archiv-Nr.: 13185 B/56, elektronenoptisch 17300 ×, Abb. 74400 ×.
Abb. 2 (*unten*). Ausschnitt einer Kupfferschen Sternzelle der Rattenleber. Osmiophiler Körper mit zahlreichen kontrastreichen Granula. Fixierung nach Sjöstrand. Aufnahme mit dem RCA EMU 2c Elektronenmikroskop. Archiv-Nr. 13035 E/56, elektronenoptisch 11200 ×, Abb. 87400 ×.

INDUCTION OF RESPIRATORY DEFICIENCY IN TETRAPLOID *SACCHAROMYCES* BY ULTRAVIOLET RADIATION[1]

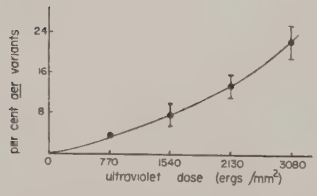
D. D. PITTMAN

Biological Research Laboratory, Southern Illinois University, Carbondale, Ill., U.S.A.

Received November 2, 1956

Raut [5] observed that over 50 per cent of haploid yeast cells surviving ultraviolet radiation formed small colonies, presumably respiratory deficient (aer).[2] It has been inferred from genetic and biochemical studies [1, 5, 6] that this deficiency, which occurs spontaneously (10^{-2}) is some haploid strains, results from the loss of cytoplasmic "particulates" rather than from gene mutation. In studies along several lines in this laboratory, aer variants were seldom encountered in populations of tetraploid yeasts, although they were frequently found among the progeny of such cells exposed to ultraviolet. This observation prompted a study of the ultraviolet dose-response curve for aer variants induced in tetraploid cells, along with some of the distinguishing characteristics of these variants.

Experimental. —Cells from 24-hour old AER tetraploid cultures of *Saccharomyces* 11294 × 11296 [2] were harvested from glucose nutrient agar slants, washed twice with $M/15$ KH_2PO_4, and resuspended in this menstruum to a concentration of 2×10^6 cells/ml. Thirty ml of the suspension were transferred to a Petri dish bottom (containing a magnetic stirrer) and irradiated with ultraviolet (95 per cent 2537 Å) at a rate of 7.7 ergs/mm²/sec (at the surface of the suspension). Yellow light was used throughout to prevent against photoreactivation. Control and irradiated samples were diluted and aliquots of the appropriate dilutions spread over the surfaces of



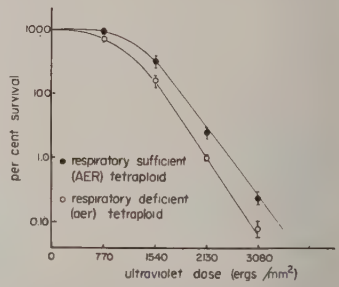


Fig. 1 (*left*). The ultraviolet dose-response curve for respiratory deficient (aer) variants induced in tetraploid yeast cells.
Fig. 2 (*right*). Ultraviolet inactivation curves of respiratory sufficient (AER) and respiratory deficient (aer) tetraploid yeast cells.

[1] This work has been supported by a research grant from the United States Atomic Energy Commission AT (11-1) 176.

[2] For convenience the respiratory sufficient and respiratory deficient phenotypes will be designated, respectively, as AER and aer.

TABLE I

The frequency of respiratory deficient (aer) variants induced in ultraviolet-irradiated haploid and tetraploid yeasts.

Dose	Experiment	Per cent aer variants	
		Haploid 8256	Tetraploid 11294 × 11296
0		6.0 (±1.5)	< .10
1540 ergs/mm²	a	11.0	4.7
	b	13.8	8.9
	c	15.2	
2130 ergs/mm²	a	18.6	10.1
	b	21.5	13.3
	c	17.8	12.1

glucose nutrient agar plates. Plates were incubated for 96 hours (30°C), after which the frequency of aer variants was determined by replica plating colonies on 1 per cent lactate nutrient agar and on glucose agar. The former medium permitted only AER cells to form colonies, while the latter supported the growth of both AER and aer cells [3]. The results below are based on five independent experiments.

Results and Discussion.—Only one aer variant was detected among several thousand colonies on control plates. Following irradiation, however, the frequency of aer colonies increased to as much as 23 per cent (Fig. 1). The possibility that this increase was due to the selection of a few radioresistant aer variants, originally in the population, was excluded since aer tetraploid cells, either spontaneous or induced, were found to be more sensitive to killing than the AER type (Fig. 2). Since the spontaneous frequency was essentially nil, the increase in aer variants with increasing doses of radiation can be considered as the minimum induction frequency. The dose-response curve for aer variants rises gradually at doses required for the logarithmic inactivation of cells (Fig. 1). The multi-component nature of the curve suggests that several radiosensitive sites must be hit to produce an aer variant. At doses higher than 3.1×10^3 ergs/mm² the curve rapidly levels off at about 30 per cent. This value is roughly half that obtained by Raut [5] with haploid cells. Although this difference may suggest that the induction frequency of aer variants is ploidy dependent, results thus far (Table I) show that, on the contrary, the induction frequency is essentially ploidy *independent* in the strains employed. This is evident when the aer haploid frequency in Table I is corrected for the spontaneous aer frequency and then compared with the aer tetraploid frequency.

The parental AER culture and fourteen aer strains selected from two experiments were used in further studies. As previously found with haploid strains [3, 4], the AER type grew well on lactate, acetate, pyruvate, and formate, whereas the aer type failed to grow on any of these substrates. Mass inocula (approx. 10^8 cells) of aer cells were spread on both lactate and formate nutrient agar in an attempt to demonstrate

reversion of aer cells to the AER phenotype. No AER colonies arose after extended incubation, demonstrating the stability of the aer phenotype and suggesting that respiratory deficiency did not result from a gene controlled block in the terminal pathway of respiration. Whereas AER cells incubated on acetate sporulation medium became packed with mitochondria and sporulated within 48 hours, aer cells failed to increase their mitochondrial content and showed no evidence of sporulation after 2 weeks. Both types, when tested for mating type response, were incapable of conjugating with haploid or diploid cells of either mating type. This observation is consistent with the previous finding [2] that cells heterozygous for mating type genes (as in the case of the tetraploid used here) are incapable of conjugating.

The radiobiological and evolutionary significance of the present finding is that ultraviolet radiation inactivates a major metabolic pathway of yeast cells, regardless of their ploidy, without subsequent death. The irreversible nature of the damage and the relative ease of its induction are not inconsistent with the hypothesis of cytoplasmic, rather than genic, damage [1], although neither of these two characteristics exclude chromosomal damage. Originally sporulating tetraploid cells, when so damaged, give rise to stable polyploid variants consigned to vegetative existence since they are incapable of either mating or sporulating. Hence, the persistence of any detrimental or "beneficial" traits induced by radiation or carried by the aer cells is *limited* to the vegetative existence of such cells and cannot be transmitted through successive meiotic generations.

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ZUR FEINSTRUKTUR DER GRANULA MENSCHLICHER GEWEBSMASTZELLEN

W. STOECKENIUS

Pathologisches Institut der Universität Hamburg, Deutschland

Eingegangen am 5. November 1956

Technik.—Fixierung in gepuffertem Osmiumtetroxyd nach Palade pH 7,4, 2 Std. Einbettung über Alkohol und Aceton in Methacrylat, Polymerisation mit 2% Benzoylperoxyd bei 48°. Schnitte auf dem Mikrotom nach Huppertz und v. Borries mit Glasmessern. Siemens-Elektronenmikroskop Typ ÜM 100e.

Bei elektronenmikroskopischer Untersuchung einer chronisch entzündeten menschlichen Tonsille fanden wir stellenweise gehäuft Gewebsmastzellen. Die spezi-

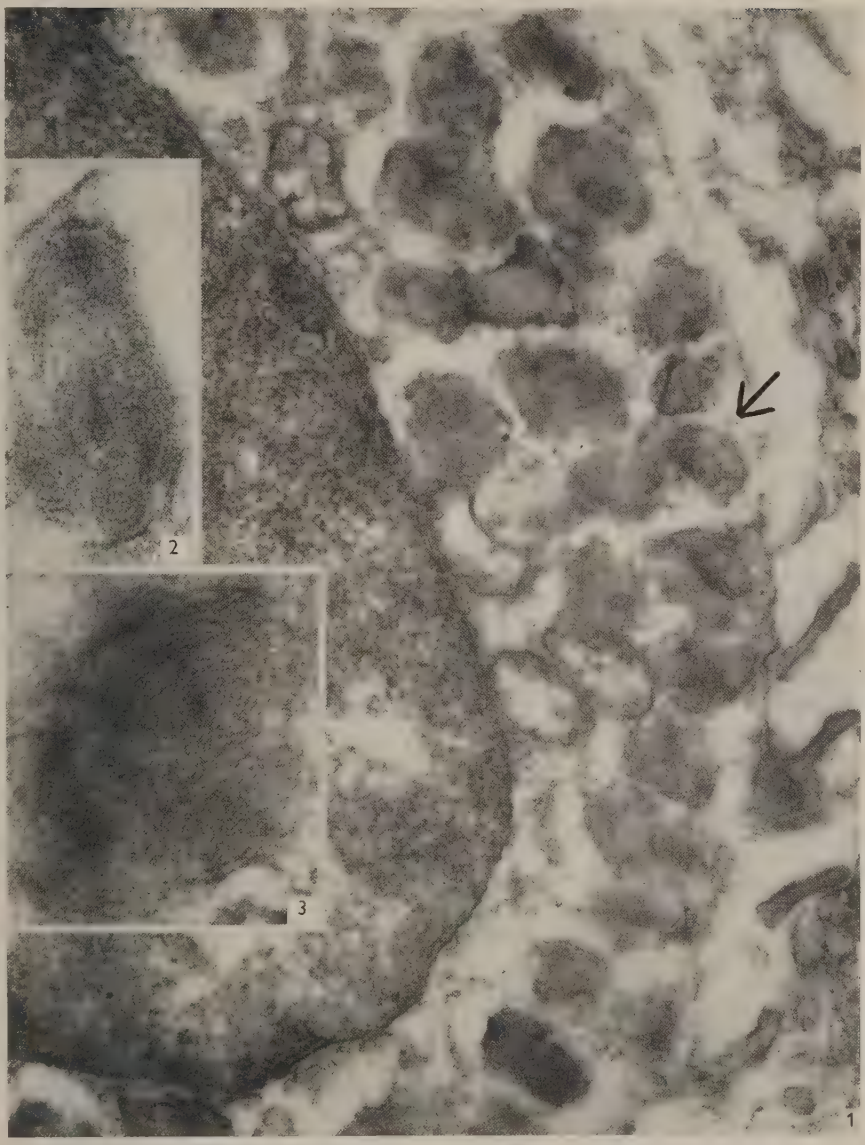

Abb. 1. Gewebsmastzelle aus einer menschlichen Tonsille. Das Plasma enthält reichlich Granula mit Lamellenstruktur und einige Mitochondrien mit wenigen kurzen Cristae. Am rechten Bildrand sind 5 der charakteristischen fingerförmigen Cytoplasmaausläufer angeschnitten. 45000 ×.
Abb. 2. Einzelnes Granulum aus einer anderen Zelle, das am linken oberen Rand die Lamellen in einem Teil ihres Verlaufes als sehr feine, scharf begrenzte Linien zeigt. 120000 ×.
Abb. 3. Ausschnitt aus Abb. 1 bei stärkerer Vergrösserung. 120000 ×.

fischen Granula dieser Zellen zeigen auch nach Osmiumtetroxyd-Fixierung und Methacrylateinbettung noch eine deutliche Metachromasie, wenn nach Herauslösen des Methacrylats mit Toluidinblau gefärbt wird. Sie sind daher in lichtoptischen Vergleichsschnitten leicht zu identifizieren. Die Grösse der annähernd runden oder ovalen Granula beträgt 0,6 bis 0,7 μ, ihre Anzahl schwankt von Zelle zu Zelle stark. Oft sind sie in der Peripherie angehäuft, während ein perinucleärer Hof freibleibt. In allen Fällen wiesen diese Granula eine charakteristische Feinstruktur aus konzentrischen, oft multizentrischen, Lamellensystem auf, die an Fingerabdrücke erinnert (Abb. 1) Die Breite der elektronenoptisch dichten Lamellen beträgt etwa 50 Å, ihr Abstand 60 120 Å. Stellenweise hat man den Eindruck, dass die Lamellen aus einem noch wesentlich dünneren, scharf begrenzten zentralen Anteil bestehen, dem sich aussen körniges, nicht so scharf begrenztes elektronendichtes Material anlagert (Abb. 2). Die Substanz zwischen den Lamellen erscheint homogen und etwas dichter als das umgebende Cytoplasma. In einem Teil der Granula nimmt diese homogene Substanz grössere Abschnitte des Granulums ein, ohne dass hier Lamellen nachweisbar wären. Andere Granula enthalten noch einen ovalen bis runden elektronenmikroskopisch dichteren Körper, der häufig eine begrenzende, äussere Membran und eine netzartige Zeichnung im Innern aufweist (Abb. 1, Pfeil).

Bloom *et al.* [1] haben die licht- und elektronenoptische Struktur von Mastzellen aus dem Mastocytom des Hundes beschrieben. Sie entspricht weitgehend unseren Beobachtungen am menschlichen Material mit den charakteristischen fingerförmigen Cytoplasmaausläufern, Mitochondrien und einzelnen unregelmässig gestalteten osmiophilen Zelleinschlüssen unbekannter Natur zwischen den Granula. In einer neueren Arbeit [2] erwähnen diese Autoren auch eine „layered structure" der Granula und bilden ein solches Granulum ab, das in einem peripheren Abschnitt einige parallele Lamellen zeigt, die den oben von uns beim Menschen beschriebenen entsprechen. Pease [3, 4] fand in den Granula basophiler Myelozyten des Meerschweinchens ebenfalls eine lamelläre Feinstruktur, doch scheinen diese Lamellen nicht konzentrisch sondern parallel zum Durchmesser der Granula angeordnet zu sein. Auch sind sie nach Fixierung in wässriger Osmiumtetroxydlösung nicht mehr mit der Giemsa-Färbung darstellbar, während unsere lichtoptischen Vergleichsschnitte unter diesen Bedingungen eine kräftige Anfärbung der Mastzellengranula zeigen. Es scheint also den bislang bekannten Unterschieden zwischen Blut- und Gewebsmastzellen auch ein Unterschied in der Feinstruktur der Granula zu entsprechen.

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THE HEAT INACTIVATION AND PRODUCTION OF CYTOCHROME DEFICIENCY IN YEAST[1]

F. SHERMAN[2]

Donner Laboratory of Biophysics and Medical Physics University of California, Berkeley, Calif., U.S.A.

Received November 7, 1956

Cytoplasmically inherited cytochrome deficient yeast cells (petites) arise spontaneously from normal cells. The rate of this mutation can be enhanced by euflavin [1], ultraviolet light [3], and growth at elevated temperatures [6]. This paper deals with the preliminary results of still another method, exposure to lethal temperatures.

The heat inactivation of several strains of *Saccharomyces cerevisiae* has been studied under various conditions. The cultures used in the experiments were prepared by spreading a dilute suspension of yeast on nutrient agar plates and incubating at 30°C. After the desired time, cells were scraped off the surface of the plates and suspended. Survival curves were obtained by diluting the suspension of yeast cells in sterile distilled water at the temperature selected for inactivation (54°C). Aliquots were withdrawn as a function of time and the heat treated cells were then plated on YED (½ per cent Difco yeast extract, 1 per cent dextrose, 2 per cent Difco bacto-agar), incubated at 30°C for several weeks, and then counted.

It was found that culture age had a great effect on the survival curve. This is in general agreement with heat inactivation of *E. coli* [2]. When cells were grown on YED they exhibited mixed sigmoid survival curves; older cultures showed greater resistance. A simple sigmoid survival curve was obtained when yeast were grown on PD (potato dextrose agar, Difco). Cultures obtained in this manner showed the greatest resistance, and in the diploid strain that was tried there was no age effect after 10 days (Fig. 1).

A cytochrome deficient petite strain was isolated from the wild type. The petite strain showed a greater heat sensitivity than the wild type (Fig. 1). It is of interest to note that this difference is not observed with ultraviolet [3] or x-ray [4] inactivation.

One day old YED cultures of haploid and diploid strains show no significant difference to heat sensitivity but higher ploidies are approximately four times more sensitive. Haploid cells grown on PD are considerably more resistant than similarly treated diploid cells (Fig. 1). The difference between these results and those of Wood [5] may have arisen from the use of different strains or culture ages.

It was found that a high percentage of the survivors of the heat inactivation develop into cytochrome deficient colonies, the fraction increasing with the percentage of cells inactivated. This result is similar to that obtained when yeast is grown at above optimum but sublethal temperatures [6], except that growth is not a necessary condition at the higher temperatures. (This latter result has been confirmed for

[1] Supported in part by the U.S. Atomic Energy Commission.

[2] Public Health Service Research Fellow of the National Cancer Institute.

these strains.) Attempts to quantitate this effect were hampered by high rate of reversion of petite colonies to normal. Also, the percentage of petite colonies was a function of the number of total colonies on the plate, a result similar to that found for ultraviolet induction of petites [3]. Since the petite mutant is unable to grow on

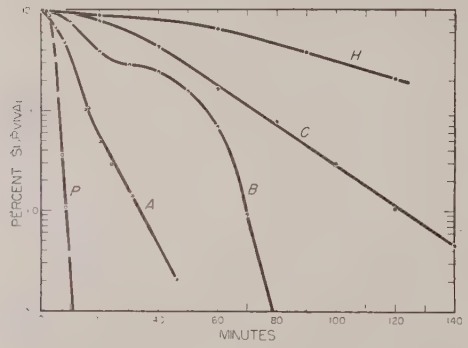

Fig. 1. The heat inactivation of several strains of *Saccharomyces cerevisiae* at 54°C. Curves *A*, *B*, and *C* are normal diploid. *A* is a one-day culture grown on YED; *B* is a 14-day culture grown on YED; *C* is a 16-day culture grown on PD. *P* is a petite diploid strain grown 3 days on YED. *H* is a normal haploid grown 16 days on PD.

a sugarless medium [3] the heat inactivated cells were streaked on nutrient agar plates with and without sugar. It was found that although there was a decrease in the number of colonies on the no-sugar plates at long exposures to heat, a corresponding increase was found at lower doses, a result difficult to explain. This effect is being studied further.

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THE ANIMALIZING EFFECT OF α-LIPOIC ACID ON SEA URCHIN EGGS

J. RUNNSTRÖM

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden and Stazione Zoologica, Naples, Italy

Received November 15, 1956

As is well known, the balance between the animal and the vegetal morphogenetic factors in sea urchin eggs may be shifted by addition of different substances, cf. [1, 3, 7–19]. The effect of the oxidized form of α-lipoic acid was tested from this point of view on developing eggs of *Paracentrotus lividus*.

Experiments.—The concentrations of α-lipoic acid were in general: *a*, 0 (control); *b*, 1×10^{-5}; *c*, 3×10^{-5}; and *d*, 6×10^{-5} *M* in sea water (pH 8.0). The eggs were brought into the solutions about three hours after fertilization. After exposure to *b*, *c* and *d* for 7–8 hours one portion of the eggs was transferred to normal sea water (*bsw*, *csw*, *dsw*), whereas another portion remained in the solutions (*b*, *c*, *d*).

There was no delay in the formation of the blastocoel in *b*–*d* as compared with *a*, the control in sea water. The formation of the primary mesenchyme was delayed. Eleven hours after fertilization this was already formed in *a;* in *b* and *c*, on the other hand, free-swimming elongated blastulae without mesenchyme were found. In *d* most of the mesenchyme-free blastulae were still enclosed in the fertilization membrane. Only in the course of the following hours did the mesenchyme cells gradually immigrate into the blastocoel. After 24 hours early plutei were observed in *a*, whereas in *b* the bilateral gastrulae predominated; about one tenth of the larvae were elongated blastulae with primary mesenchyme, the cells of which were bilaterally or irregularly arranged. In *c* similar mesenchyme blastulae predominated and in *d* they constituted the whole material. The series *bsw*, *csw*, *dsw* differed but slightly from *a*, *b* and *c*.

After 46 hours the situation was the following: *a*, well developed plutei with stomodeum; *b*, plutei smaller than in the control, with convergent apical skeleton rods. In about 5 per cent of the larvae there was exogastrulation, but no stomodeum. In *bsw* the plutei were slightly smaller than in the control and all the larvae were with stomodeum. In *c* there was about 10 per cent small plutei, 90 per cent exogastrulae without differentiation of coelom rudiment; the ectoderm had an enlarged oral field, the skeleton being rudimentary but in general bilateral; no stomodeum in the exogastrulae. *csw* was like *c*. In *d* animalization was pronounced with no differentiation of the endoderm; the animal part of the larva was spherical but the vegetal often drawn out into a tip. The acron with the ciliary tuft expanded so as to cover about $\frac{1}{8}$ of the surface of the larva; irregularly arranged primary mesenchyme, skeleton rudimentary or suppressed. In *dsw* the larvae had a more pronounced bilateral symmetry than in *d;* rudimentary entoderm which may be in- or exovaginated; no coelom rudiment; and ectoderm with enlarged acron and oral field. The primary mesenchyme was bilaterally arranged; the skeleton represented by undifferentiated spicules.

From this it follows that α-lipoic acid exerts an animalizing action on the developing egg of *Paracentrotus lividus*. An exposure during the period 3 10 hours after fertilization sufficed to stabilize the tendency toward animalization, at least in the higher concentrations of which 6×10^{-5} *M* approached those which have a toxic effect. There was a characteristic delay in the formation of the primary mesenchyme; a certain regulation towards normal morphological conditions prevailed. The extent to which this regulation occurred depended on the concentrations and time of exposure to the reagent as exemplified in the experiment quoted.

Even more striking is the animalizing effect of α-lipoic acid when combined with lithium. It is convenient to refer to an experiment parallel to that already described, and carried out on the same egg material. The tests are designated: a_{Li}, b_{Li}, c_{Li}. They all contained LiCl, 0.022 *M* (8.5 ml isot. LiCl + 91.5 ml sea water) but b_{Li}–d_{Li} in addition respectively 1, 3 and 6×10^{-5} *M* oxidized α-lipoic acid. $a_{Li}sw$ etc. means that the eggs had been transferred to normal sea water 10 hours after fertilization (7 hours exposure to the Li-containing solutions). As in the parallel experiment one

part of the eggs remained in the Li-containing solutions. No detailed report of the development of the Li-treated larvae will ge given here. The lithium effect was strongly modified already by the presence of 1×10^{-5} *M* α-lipoid acid, as shown by the follow-in distributions of the 46-hour-old larvae.

	Exogastrulation, entomesoderm occupies > 1/2 of the larvae	Partial exogastrulation, entomesoderm, occupies about 1/2 of the larvae	Entomesoderm somewhat enlarged but wholly invaginated. No arms	Number counted
$a_{\mathrm{LI}}{}^{sw}$	72.5 per cent	10.5 per cent	17.0 per cent	417
$b_{\mathrm{LI}}{}^{sw}$	17.5	48.0	34.5	437

In $a_{\mathrm{Li}}sw$ a structural modification of the cells occurred in the oesophagus region. The cells which first had the form of relatively low cylinders elongated in radial direction. Phase contrast microscopy demonstrated the presence of refringent structures orientated in the direction of elongation. They probably represented the mitochondria. After pretreatment with α-lipoic acid the described structural changes were found to occur also in the region of the midgut and sometimes also in that of the endgut. The elongation was the expression of a tendency for invagination of the cells, which was realized under favorable conditions.

In c_{Li}, d_{Li}, $c_{\mathrm{Li}}sw$ and $d_{\mathrm{Li}}sw$ the lithium effect was not only compensated but the tendency for animalization predominated to an extent that increased with the concentration of the α-lipoic acid. The entomesoderm appeared as a cell plate which sometimes was slightly concave. In other cases the entomesoderm invaginated and and the entoderm differentiated into an intestine with its three compartments. The acron and ciliary tuft were enlarged in the same larvae. Within the entomesoderm region smaller protrusions were often found. Upon closer examination these proved to consist of ectoderm. They attracted mesenchyme cells which sometimes formed minute pieces of skeleton, cf. [14]. The extra ectoderm zones within the entomesoderm appeared also in a_{Li} and $a_{\mathrm{Li}}sw$ but here they were found only to the extent of 0.5 per cent whereas after the combined treatment with α-lipoic acid and lithium a high proportion of the larvae, 20–30 per cent, carried the extra ectoderm protrusions. In other experiments a greater number of ectoderm protrusions was found even after mere lithium treatment.

Animalization depresses the formation of the red pigment which normally appears after the gastrulation [2]. In the reported experiment the number of cells with red pigment was counted in 25 46-hour-old larvae from each of the tests after fixation in 4 per cent formalin. The following average values were found:

$a_{\mathrm{LI}}{}^{sw}$	$b_{\mathrm{LI}}{}^{sw}$	$c_{\mathrm{LI}}{}^{sw}$	$d_{\mathrm{LI}}{}^{sw}$
28.4	12.5	6.6	2.2

In a non-lithium-treated control of the same age and from the same material the average number of pigment cells amounted to 29.4.

The α-lipoic acid used in the experiment quoted above is not 100 per cent oxidized. It consumed some added porphyrindin. In order to keep the α-lipoic acid (10^{-5} *M*)

at a higher oxidation reduction state, 2.8×10^{-5} *M* porphyrindin was added. The eggs were immersed in sea water containing these reagents 3½ hours after fertilization. The animalizing effect was more pronounced in the mixture of α-lipoic acid and porphyrindin than in α-lipoic acid alone. After exposure to the reagent for 20 hours the eggs were transferred to normal sea water, fixed and counted 12 hours later. In 42 per cent of the larvae subjected to the combined pretreatment no invagination of entoderm occurred, whereas in those pretreated with α-lipoic acid alone only 13 per cent were without entoderm invagination. If reduced α-lipoic acid was used in the experiment the number of larvae without entoderm invagination was only about 3 per cent (about 350 larvae counted in each test). The reduced α-lipoic acid was stabilized by addition of 6.5×10^{-4} *M* glutathion. Even the reduced α-lipoic acid gave a certain animalization, but definitely to a lesser extent than the oxidized form. After pretreatment with the reduced form the stomodeum anlage was often reinforced. This should rather indicate enhanced activity of the vegetal factors [4, 5].

It was demonstrated that a decrease of respiration favours the vegetalizing action of lithium [8, 14]. This led to the opinion that the vegetal part represents a region where a more reduced state prevails as compared with the animal part [18]. In connection with the present work it was also demonstrated that glutathion, ascorbic acid and sodium azide combined with lithium in sea water gave a stronger vegetalization than lithium alone. In one experiment the lithium concentration was 0.03 *M* and that of ascorbic acid 0.003 *M*. The exposure to sea water with lithium alone (*a*) or with lithium and ascorbic acid (*b*) started 3½ hours and lasted until 11 hours after fertilization. Thereafter the eggs were transferred to normal sea water (*asw*, *bsw*); 52 hours after fertilization the material was distributed in the following way:

	Vegetalization. Total exogastrulation	Less pronounced vegetalization. Partial exogastrulation	Plutei. Endogastrulation	Number counted
a sw	9 per cent	13 per cent	78 per cent	345
b sw	83	7	10	284

When applied without lithium the agents had at most a very weak vegetalizing action, manifested by a somewhat narrower acron in a typical pluteus.

Discussion.—The results presented seem to be in keeping with the view that differences with respect to oxidation reduction state are involved in the animal-vegetal balance [14, 16, 18], a view which still may have a heuristic value. The transplantation of micromeres into a more animal region not only demonstrates their capacity of morphogenetic induction [4, 5]: primarily a reduction center is created owing to inducing factors emanating from the micromeres. Probably the increased reduction capacity is due to an accumulation of hydrogen donors which find no sufficient outlet through the acceptor systems of the mitochondria. The structural effect of lithium [13] favors an enlargement of the vegetal region by quenching the outlet of hydrogen from the donors. On this assumption a decrease in respiration [8, 14] or addition of reducing systems should accentuate the lithium effect whereas addition of oxidizing systems or exposure to pure oxygen [11] should counteract the effect, which is in agreement with the evidence at hand.

The preceding considerations already reveal that there are several different steps in the differentiation process. One should therefore not expect to find a full uniformity in the character of the substances which have an animalizing effect, cf. [10, 11, 12]. They may in different ways interfere with the competition between vegetal and animal factors [15] or finally cause a direct change in the vegetal physiological conditions which are established during the early development, cf. [15].

The stabilization of the determination starts with the mesenchyme. A full animalization with suppression even of the mesenchyme was obtained only by pretreatment of the eggs before fertilization [7, 17, 18]. In larvae animalized by treatment applied to developing eggs mesenchyme was always formed to some extent.

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THE INCORPORATION OF C^{14}-L-LEUCINE INTO RAT LIVER PROTEINS *IN VITRO* VISUALIZED AS A TWO-STEP REACTION

T. HULTIN and GUNILLA BESKOW

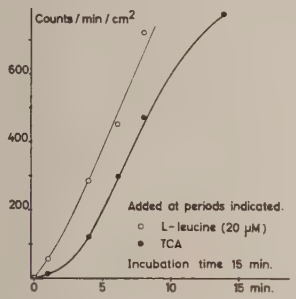
The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Received November 15, 1956

In a previous note [4] a few experiments were described suggesting that the incorporation of C^{14}-glycine into rat liver proteins *in vitro* is accomplished in two successive steps, demonstrable by diluting the incorporation system at different periods with unlabeled glycine in large excess. Since C^{14}-glycine in liver systems is rapidly incorporated also into glutathione [5], and it has been shown that labeled glutathione

may interfere with isotope determinations in the protein fraction [6], it seemed essential to study the incorporation of a different amino acid under similar conditions.

Experimental.—Male albino rats (150–175 g) were used. Liver homogenates, freed from cell fragments and mitochondria by centrifugation for 5 minutes at 12,000 *g*, were prepared, essentially according to the method of Zamecnik and Keller (8), $MgCl_2$



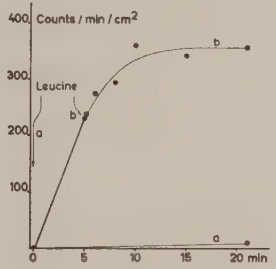
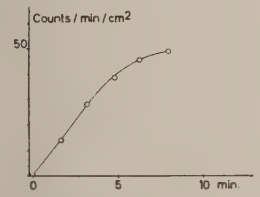


Fig. 1 (*left*). Incorporation of C^{14}-L-leucine into the proteins of a mitochondria-free rat liver system. Effects of isotope dilution at different periods of incubation.
Fig. 2 (*right*). Incorporation system of the same kind as in Fig. 1. Non-labeled leucine (20 μM per sample) was added to the system (a) at zero time, (b) after 5 minutes of incubation. TCA was added at the periods indicated.



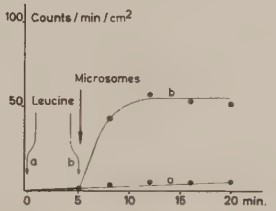


Fig. 3 (*left*). The incubation started with an incorporation system containing cell fluid freed from microsomes. At the periods indicated non-labeled leucine (20 μM) was added to the samples, followed within a few seconds by standard amounts of microsomes. TCA was added to each sample 15 minutes after addition of the microsomes.
Fig. 4 (*right*). Incorporation system of the same kind as in Fig. 3. Isotope dilution was performed (a) at zero time, (b) after 5 minutes of incubation. Standard amounts of microsomes were in both cases added after 5 minutes of incubation. TCA was added at the periods indicated.

(0.01 *M*), potassium phosphate (0.02 *M*, pH 7.8.), $KHCO_3$ (0.03 *M*) KCl (0.025 *M*), and sucrose (0.35 *M*) being included in the homogenization medium. In some experiments the microsomes and the cell fluid fraction were separated by centrifugation at 105,000 *g* for 45 minutes. The isolated microsomes were suspended by gentle homogenization in 1/6 volume of the same homogenization medium. Of the homogenates 0.7 ml samples were incubated at 30°C in final volumes of 1 ml with 0.02 μM of C^{14}-L-leucine (6 mc/m *M*) in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. In the case of the isolated fractions 0.6 ml of cell fluid and 0.1 ml

of suspended microsomes were incubated under similar conditions. Phosphoenolpyruvate (10 μM) and ATP (1 μM) were included in the systems as the energy source. The phosphoenolpyruvate was synthesized according to Baer [1]. The ATP was a Sigma preparation.

After the incubations the samples were treated with L-leucine in large excess, and the proteins were precipitated with trichloroacetic acid (TCA). The protein samples were purified by the method of Schneider [7]. The activities of the protein samples were recalculated for indefinite sample thickness (1 cm^2 plate area) on the basis of an empirical activity saturation curve.

Effects of isotope dilution at different periods.—At successive periods after the start of the incorporation experiments a 1000-fold isotope dilution was effected in the incorporation system by the addition of 20 μM of unlabeled L-leucine per sample. When the isotope dilution was carried out at the start of the experiments the subsequent incorporation of isotopic leucine into proteins became almost entirely suppressed. When, on the other hand, the unlabeled leucine was added to the system some minutes later, the isotope content of the proteins continued to increase for 5–10 minutes (Figs. 1, 2).

Cell fluid samples, freed from microsomes, were used in incorporation experiments. Very low incorporation values were observed. When the proper amounts of suspended microsomes were added to such samples, a rapid incorporation of C^{14}-leucine into the proteins was initiated. To incorporation systems containing cell fluid freed from microsomes, unlabeled L-leucine (20 μM per sample) was added at different periods of incubation, followed within a few seconds by the suspended microsomes. When these additions were made at the start of the incubation, very low incorporation values were recorded. When, instead, the same additions were made some minutes later, a significant incorporation was initiated by the microsomes, lasting for 5–10 minutes (Figs. 3, 4).

The experiments suggest that labeled intermediates, not very rapidly equilibrating with the leucine pool, temporarily accumulate in the incorporation systems during incubation. The intermediates may subsequently be incorporated into proteins. A formation of intermediates may take place even in microsome-free cell fluid systems under the conditions described.

These postulated intermediates may be related to the hydroxylamine-binding amino acid derivatives which are formed in energy-supplied liver systems under the conditions studied by Hoagland *et al.* [2, 3, 9].

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